THIS PART COMPLETES VOLUME 72 (1959)
AUGUST 1959, VOLUME 72, No. 4

THE UNIVERSITY OF MICHIGAN
OCT 1 1959
SCIENCE LIBRARY

THE BIOCHEMICAL JOURNAL

Edited for

THE BIOCHEMICAL SOCIETY

Editorial Board

A. G. OGSTON (*Chairman*) — H. J. ROGERS (*Deputy Chairman*)
E. P. ABRAHAM — D. C. HARRISON
W. N. ALDRIDGE — J. LASCELLES
W. BARTLEY — L. W. MAPSON
E. A. DAWES — R. MARKHAM
R. M. C. DAWSON — R. J. PENNINGTON
G. E. FRANCIS — J. J. SCOTT
Q. H. GIBSON — W. V. THORPE
C. H. GRAY — W. J. WHELAN

Secretary to the Board: F. CLARK

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W. 1
AMERICAN BRANCH: 32 EAST 57TH STREET, NEW YORK 22, N.Y.

Price 25s. net (U.S.A. $4.25)

The *Biochemical Journal* is conducted by the **Biochemical Society** and is published by the Cambridge University Press. The *Journal* is issued in three volumes per year. Each volume consists of approximately 700 pages, published in four parts at intervals of one month. The index for each volume is issued separately.

The Biochemical Society was instituted solely for the advancement of the Science of Biochemistry, and holds meetings at different centres for the communication of original papers, and for the discussion of current problems. Persons interested in Biochemistry are eligible for election. Members whose subscriptions are not in arrears are entitled to receive the *Biochemical Journal* without further payment. Further information may be obtained from the Honorary Secretaries, Dr C. E. Dalgliesh, Postgraduate Medical School of London, Ducane Road, Hammersmith, London, W. 12, and Dr P. N. Campbell, The Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1, or from the Honorary Treasurer, Dr F. A. Robinson, c/o Allen and Hanburys, Ltd., Bethnal Green, London, E. 2.

Subscriptions to the *Biochemical Journal*. For non-members of the Biochemical Society the subscription to the *Biochemical Journal* is £4 per volume, payable in advance to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, or to any bookseller. In the United States of America the *Journal* may be purchased from the Cambridge University Press American Branch, 32 East 57th Street, New York, 22 (subscription price $13.50 per volume).

Communications respecting the sale of single issues or back numbers of the *Journal*, Volumes 20–72, should be addressed to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, from whom a list of prices is available. Volumes 1–15 have been reproduced and are available from William Dawson & Sons, Ltd., 4 Duke Street, Manchester Square, London, W. 1.

Claims for the replacement of *Journals* lost in transmission will not be entertained if they are received later than three months after the date of the posting of the *Journal*.

The following are available in paper covers:

Volumes 20–28.
Volume 29 Nos. 1, 2, 4, 6, 8–11.
Volume 33 Nos. 8–12, Index.
Volume 34 Nos. 4–7 & 12.
Volume 34 Nos. 8 & 9, 10 & 11.
Volumes 35–38.
Volume 39 Nos. 2 & 3.
Volumes 40–47.
Volumes 50–58.
Volumes 60–72.
Index to Volumes 11–20.
Index to Volumes 21–30.
Index to Volumes 31–40.
Index to Volumes 41–65.
History of the Biochemical Society.

Reduced terms for Volumes 1–15 are available to members of the Biochemical Society, who should apply to the Honorary Secretaries.

Nucleoti
A study
The alka
By
The poi
Bai
Biochem
But
The occ
Ros
1-(*o*-Carb
and
Intermo
solu
Thetin–
sulp
An expl
iodo
Inhibitio
The proc
By
Carbon
grow
Carbon
free
Ion tran
Bre
The proc
By
The inco
on t
Observa
I. C
Oxidativ
plat
Selective
and
A ribonu
Two-way
follo
The isol
(Wi
The reac
Bla
Nucleosi

CONTENTS OF VOLUME 72, No. 4, 1959

(*All rights reserved*)

PAGE

Nucleotides of *Hevea brasiliensis* Latex. A ribonucleoprotein component. By A. I. McMullen . . 545

A study of protein-binding in the metabolism of vitamin B_{12}. By M. E. Gregory and E. S. Holdsworth 549

The alkaloids of hemlock (*Conium maculatum* L.). Distribution in relation to the development of the fruit. By J. W. Fairbairn and S. B. Challen 556

The point of the aerobic inhibition of glycolytic activity associated with brain mitochondria. By R. Balázs 561

Biochemistry of sheep tissues. Enzyme systems of liver, brain and kidney. By C. H. Gallagher and S. H. Buttery 575

The occurrence of guanidinoacetic acid and other substituted guanidines in mammalian liver. By H. Rosenberg 582

1-(*o*-Carboxyphenylamino)-1-deoxyribulose. A compound formed by mutant strains of *Aerobacter aerogenes* and *Escherichia coli* blocked in the biosynthesis of tryptophan. By C. H. Doy and F. Gibson . . 586

Intermolecular and intramolecular reactions of human-serum albumin after its X-irradiation in aqueous solution. By D. Rosen 597

Thetin–homocysteine transmethylase. The distribution of the enzyme, studied with the aid of trimethylsulphonium chloride as substrate. By G. A. Maw 602

An explanation for the difference between the responses of mammals and birds to thyroxine and triiodothyronine. By J. R. Tata and C. J. Shellabarger 608

Inhibition of aconitase by glyoxylate plus oxaloacetate. By A. Ruffo, M. Romano and A. Adinolfi . 613

The production of pyruvic acid, oxaloacetic acid and α-oxoglutaric acid from glucose by tissue in culture. By G. A. Abdel-Tawab, E. Broda and G. Kellner 619

Carbon assimilation by *Pseudomonas oxalaticus* (OX 1). 1. Formate and carbon dioxide utilization during growth on formate. By J. R. Quayle and D. B. Keech 623

Carbon assimilation by *Pseudomonas oxalaticus* (OX 1). 2. Formate and carbon dioxide utilization by cell-free extracts of the organism grown on formate. By J. R. Quayle and D. B. Keech . . . 631

Ion transport and metabolism in slices of guinea-pig seminal-vesicle mucosa. By R. Whittam and H. J. Breuer 638

The products of the partial acid hydrolysis of the mucopeptide from cell walls of *Micrococcus lysodeikticus*. By H. R. Perkins and H. J. Rogers 647

The incorporation of amino acids into the cell-wall mucopeptide of staphylococci and the effect of antibiotics on the process. By J. Mandelstam and H. J. Rogers 654

Observations on the composition and metabolism of the nitrogen-fixing root nodules of *Myrica*. By G. Leaf, I. C. Gardner and G. Bond 662

Oxidative enzyme systems in ossifying cartilage. By R. G. Whitehead and S. M. Weidmann. (With one plate). 667

Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep. By R. F. Glascock and W. G. Hoekstra 673

A ribonucleoprotein of skeletal muscle and its relation to the myofibril. By S. V. Perry and M. Zydowo 682

Two-way separation of amino acids and other ninhydrin-reacting substances by high-voltage electrophoresis followed by paper chromatography. By M. L. Efron 691

The isolation and characterization of acetylcholine-containing particles from brain. By V. P. Whittaker. (With one plate) 694

The reaction of tetrahydropteroylglutamic acid and related hydropteridines with formaldehyde. By R. L. Blakley 707

Nucleoside diphosphokinase of pea seeds. By R. J. A. Kirkland and J. F. Turner 716

Proceedings of the Biochemical Society

PAGE

Stereochemical correlations. By W. KLYNE 39P

Steric aspects of the biosynthesis of terpenes and steroids. By D. ARIGONI 39P

Steric aspects of drug action. By R. B. BARLOW 40P

cis-trans Isomers of retinene in visual processes. By G. A. J. PITT and R. A. MORTON 40P

Steric factors in enzyme action: hydrolytic enzymes. By E. C. WEBB 41P

Steric factors in enzyme action: oxidation–reduction enzymes. By E. C. SLATER 41P

Stereochemical specificities of some enzymes of nucleotide metabolism. G. R. BARKER 42P

INDEX OF AUTHORS

	PAGE
Abdel-Tawab, G. A.	619
Adinolfi, Anna	613
Arigoni, D.	39P
Balázs, R.	561
Barker, G. R.	42P
Barlow, R. B.	40P
Blakley, R. L.	707
Bond, G.	662
Breuer, H. J.	638
Broda, E.	619
Buttery, S. H.	575
Challen, S. B.	549
Doy, C. H.	586
Efron, Mary L.	691
Fairbairn, J. W.	556
Gallagher, C. H.	575
Gardner, Isobel C.	662
Gibson, F.	586
Glascock, R. F.	673
Gregory, Margaret E.	549
Hoekstra, W. G.	673
Holdsworth, E. S.	549
Keech, D. B.	623, 631
Kellner, G.	619
Kirkland, Ruth J. A.	716
Klyne, W.	39P
Leaf, G.	662
McMullen, A. I.	545
Mandelstam, J.	654
Maw, G. A.	602
Morton, R. A.	40P
Perkins, H. R.	647
Perry, S. V.	682
Pitt, G. A. J.	40P
Quayle, J. R.	623, 631
Rogers, H. J.	647, 654
Romano, Marta	613
Rosen, D.	597
Rosenberg, H.	582
Ruffo, A.	613
Shellabarger, C. J.	608
Slater, E. C.	41P
Tata, J. R.	608
Turner, J. F.	716
Webb, E. C.	41P
Weidmann, S. M.	667
Whitehead, R. G.	667
Whittaker, V. P.	694
Whittam, R.	638
Zydowo, M.	682

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*:

Steps in the oxidation of xanthine to uric acid catalysed by milk xanthine oxidase. By H. Gutfreund and J. M. Sturtevant

The activation of an arylsulphatase from ox liver by chloride and other anions. By E. C. Webb and Patricia F. W. Morrow

The mechanism of action of chlorpromazine. 2. Reduced diphosphopyridine nucleotide–cytochrome *c* reductase and coupled phosphorylation. By M. J. R. Dawkins, J. D. Judah and K. R. Rees

Incorporation of DL-[1-^{14}C]leucine and [1-^{14}C]*iso*valeric acid into milk constituents by the perfused cow's udder. By R. Verbeke, Monique Lauryssens, G. Peeters and A. T. James

Isolation of lombricine and its enzymic phosphorylation. By Radha Pant

Pepsin-catalysed transpeptidation of the amino-transfer type. By Hava Neumann, Y. Levin, A. Berger and E. Katchalski

Biochemical adaptation as a response to exercise:

1. Effect of swimming on the levels of lactic dehydrogenase, malic dehydrogenase and phosphorylase in muscles of 8-, 11- and 15-weeks-old rats. By M. K. Gould and W. A. Rawlinson

2. Adenosine triphosphatase and creatine phosphokinase activity in muscles of exercised rats. By W. A. Rawlinson and M. K. Gould

Enzymes of the pentose phosphate pathway in the mung-bean seedling. By Maharani Chakravorty and D. P. Burma

The metabolism of exogenous ^{14}C-labelled 5-hydroxytryptamine in rats. By Dina Keglević, Z. Supek, S. Kveder, Sonja Iskrić, S. Kečkeš and Alemka Kisić

The effect of hypophysectomy of the rat and of treatment with growth hormone on the incorporation of amino acids into liver proteins in a cell-free system. By A. Korner

Incorporation of sodium [1-^{14}C]hexanoate and sodium hydrogen [^{14}C]carbonate into milk constituents by the perfused cow's udder. By Monique Lauryssens, R. Verbeke, G. Peeters and Agnes Donck

Parapepsins. Two proteolytic enzymes associated with porcine pepsin. By A. P. Ryle and R. R. Porter

The denaturation of ovalbumin. Changes in optical rotation, extinction and viscosity during serial denaturation in solutions of urea. By F. S. Steven and G. R. Tristram

Kinetics and equilibria in systems containing haem, carbon monoxide and pyridine. By M. H. Smith

The preparation and some properties of myoglobin containing meso- and deutero-haem. By M. H. Smith and Q. H. Gibson

The lipids of whole blood. 1. Lipid biosynthesis in human blood *in vitro*. By A. T. James, J. E. Lovelock and Joan P. W. Webb

The localization of ^{131}I in biosynthetically labelled [^{131}I]thyroxine. By L. G. Plaskett

The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. By R. R. Porter

The *N*-acetylation and estimation of hexosamines. By G. A. Levvy and A. McAllan

Studies on the phosphoproteins of brain: the intracellular localization in brain of a phosphoprotein involved in the metabolic response of cortical slices to electrical stimulation. By P. J. Heald

Comparative studies of 'bile salts'. 12. Application to a problem of rodent classification: bile salts of the cutting-grass, *Thryonomys swinderianus*. By G. A. D. Haslewood and A. U. Ogan

Studies of sebum. 9. Further studies of the composition of the unsaponifiable matter of human-forearm 'sebum'. By Barbara Boughton and V. R. Wheatley

The inhibition of bacteriophage multiplication by proflavine and its reversal by certain polyamines. By D. KAY

The differential effect of arginine and canavanine on growth and enzyme formation in *Staphylococcus aureus* 524 SC. By M. H. RICHMOND

The spectrophotometric determination of protein at 210 mμ. By M. P. TOMBS, FELICITY SOUTER and N. F. MACLAGAN

Some properties of uterus actomyosin and myofilaments. By D. M. NEEDHAM and J. M. WILLIAMS

The chemistry of xanthine oxidase:

4. The problems of enzyme inactivation and stabilization. By F. BERGEL and R. C. BRAY

5. Electron-spin resonance of xanthine oxidase solutions. By R. C. BRAY, B. G. MALMSTRÖM and T. VÄNNGÅRD

Metabolism of xylose by the lens. 2. Rat lens *in vivo* and *in vitro*. By RUTH VAN HEYNINGEN

THE BIOCHEMICAL JOURNAL

NOTES FOR CONTRIBUTORS

Papers submitted for publication in the *Biochemical Journal* should be written concisely. The sections below concerning the preparation of the typescript give only general indications. Authors are urged to consult the more detailed *Suggestions to Authors, Chemical Nomenclature and Abbreviations, Symbols, Usages and Conventions,* which was published in May 1957 (*Biochem. J.* 1957, 66, 1); also *Notes on Preparation of Illustrations,* which was published in January 1956 (*Biochem. J.* 1956, 62). Copies of these two pamphlets may be obtained from the Editorial Office (*Biochemical Journal*), The Lister Institute, Chelsea Bridge Road, London, S.W. 1, price 1*s.* 6*d.* (*Suggestions to Authors*) and 1*s.* (*Notes on Preparation of Illustrations*) post free.

Strict observance of the requirements of the *Biochemical Journal* will shorten the period between the receipt of a paper and its publication.

Communications. Papers submitted for publication should be sent to the Secretary to the Editorial Board (*Biochemical Journal*), The Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1.

Abstracts. Authors are requested to submit with their typescript an abstract suitable for inclusion in *International Abstracts of Biological Sciences.* This abstract will not appear in the *Biochemical Journal* but will be edited before being passed for publication in the *Abstracts.*

The abstract should outline as briefly as possible the results and definitive conclusions of the work submitted. Details of methods are generally not required. A paper of average length should be abstracted in about 100 words. The abstract should be typed in double spacing on a separate quarto sheet in the following form: title; name(s) of author(s); *Biochem. J.* (space for year, volume and page reference); address (for reprint applications); abstract. For example:

> The metabolism of short-chain fatty acids in the sheep. 4. The pathway of propionate metabolism in rumen epithelial tissue. R. J. Pennington and T. M. Sutherland. *Biochem. J.* 1956, **63**, 618–628 (Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland)—(Abstract).

Reprints. Where at least one author of a paper is a member of the Biochemical Society, twenty-five reprints are supplied free of cost. An author may purchase additional reprints if he notifies the Press on the appropriate form immediately the proof of the paper is received. Communications about the purchase of reprints should be addressed to the University Press, Cambridge.

General. It is the policy of the *Biochemical Journal* to publish papers in all fields of biochemistry—plant, animal and microbiological—provided that they describe results which make a new and fundamental contribution to biochemical knowledge, or that they describe methods applicable to biochemical problems. Submission of a paper to the Editorial Board will be held to imply that it reports unpublished work, that it is not under consideration for publication elsewhere, and that if accepted for the *Biochemical Journal* it will not be published elsewhere in the same form, either in English or in any other language, without the consent of the Editorial Board.

Contributors who reside abroad may nominate somebody in Great Britain who is willing to correct the proofs of their papers. Proofs are also sent to all authors residing abroad, if necessary by airmail, whether or not they have nominated a proof reader

p.t o.

in Great Britain; if these are returned immediately, it will normally be possible to incorporate corrections in the final proof. The method of correcting proofs given in B.S. 1219 or B.S. 1219C (obtainable from the British Standards Institution) is preferred.

Papers should be headed by an informative title, by the names of the authors and by the name and address of the Laboratory where the work was performed. Female authors should use one given name and the surname, and male authors should use initials and surname only. Descriptive material about the author, e.g. Beit Memorial Fellow, or details of financial support, should appear as a footnote on the first page or, preferably, in the acknowledgements at the end of the paper.

Typescripts should bear the name and address of the person to whom the proof of the paper is to be sent, and should give also a shortened version of the title, not exceeding forty-five letters and spaces in length, suitable for a running title in the published pages of the work.

If a paper that has been returned to an author for revision is not resubmitted within six months, it will be deemed to be a new paper and the date of receipt altered accordingly. A revised paper containing a significant amount of new material will also be redated.

A paper should be written only when a piece of work is rounded off. Preliminary or abortive experiments should not be described.

It would help the editors if the author, when submitting a paper which is part of a series, would enclose reprints of the immediately preceding parts.

Form of papers submitted for publication. Papers should be in double-spaced typing on sheets of uniform size with wide margins. Top copies only should be submitted. The paper should be written in English.

The onus of preparing a paper in a form suitable for sending to press lies in the first place with the author, who should first consult the detailed *Suggestions to Authors, Chemical Nomenclature and Abbreviations, Symbols, Usages and Conventions.* Authors should also refer to a current issue of the *Journal* in order to make themselves familiar with the typographical conventions, use of cross-headings, lay-out of tables, citation of references, etc. The need for editorial revision of a badly prepared typescript will lead to delay in publication. Papers on specialized subjects should be presented so that they are intelligible to the ordinary reader of the *Journal*. Sufficient information should be included to permit repetition of the experimental work.

Generally, papers should be divided clearly into sections, as follows: (*a*) introduction, containing the reasons for doing the work; (*b*) Experimental methods: with chemical papers the experimental part may appear towards the end, but otherwise should follow the introduction; (*c*) Results: these should be given concisely; the use of both tables and figures to illustrate the same results will only rarely be permitted; only illustrative protocols should be included; (*d*) Discussion: it is desirable that the presentation of the results should be separated from the discussion of their significance; this section should be strictly limited to discussion, and should not recapitulate results; (*e*) a Summary, about 3% of the length of the paper: the paragraphs of the Summary should be numbered; (*f*) acknowledgements; (*g*) References. The arrangement suggested for sections (*b*)–(*d*) is not binding on authors; other ways of arrangement are sometimes more suitable.

The *Biochemical Journal* uses as a standard for spelling the *Concise Oxford Dictionary of Current English* (Oxford: Clarendon Press).

Illustrations. Diagrams that do not conform with the directions given in *Notes on Preparation of Illustrations* may have to be redrawn by the Press and the expense charged to the author. Legends and captions should be written so that the general meaning of each illustration can be understood without reference to the text, and so that the exact experimental conditions used to obtain the results illustrated are made clear. Illustrations requiring reproduction as half-tone plates should be avoided whenever possible. Photographs or drawings of paper chromatograms, particularly one-dimensional, are not generally published.

Tables. Tables should have headings which make their general meaning comprehensible without reference to the text. Conditions specific to the particular experiment should be stated. Reference to the text for general experimental methods is permissible provided that there is no ambiguity. The units in which the results are expressed, e.g. g./100 ml., should be given at the top of each column, and not repeated on each line of the table.

Tables should be typed on separate sheets and their approximate position in the text indicated. Words or numerals should be repeated on successive lines: 'ditto' or ',,' are not to be used.

Footnotes. These should be avoided in the text as far as possible.

CORRIGENDUM

Intermediates in the biosynthesis of bacterial penicillinase

By M. R. Pollock and M. Kramer

Volume 70 (1958)

p. 669, column 1, line 20

for 0·31 μg. *read* 3·1 μmg.

Nucleotides of *Hevea brasiliensis* Latex

A RIBONUCLEOPROTEIN COMPONENT

By A. I. McMULLEN
The British Rubber Producers' Research Association, Welwyn Garden City, Herts

(*Received* 18 *December* 1958)

Natural rubber latex is obtained from the tree *Hevea brasiliensis* by making an incision in the bark extending in depth to within about 1 mm. of the cambial tissue (see Riches & Gooding, 1952). The latex which flows from the cut is believed to consist mainly of the content of the laticiferous-vessel system (Ruinen & de Haan-Homans, 1950). The collected latex is a suspension of several particulate phases in an aqueous serum and can be separated into fractions by high-speed centrifuging (Cook & Sekhar, 1953). The rubber particles form the uppermost layer; the 'bottom fraction' consists of sedimented material, while the intermediate layer, termed 'C-serum', represents the aqueous phase. Bottom fraction includes the 'lutoid particles' described by Homans & van Gils (1948); it is highly hydrated and contains a variety of non-rubber metabolites. The present work describes the isolation and characterization of a nucleic acid which is present as a ribonucleoprotein in C-serum and bottom fraction.

EXPERIMENTAL

The collection, centrifugal separation and freeze-drying of all the latex fractions used in the present work were carried out by the staff of the Rubber Research Institute of Malaya. The source of supply was a group of twenty mature mixed clonal seedlings growing at the Experiment Station of the Institute. These trees were tapped on a half-spiral, alternate daily system (cf. Riches & Gooding, 1952) and the latex was collected in aluminium cups at ambient temperature for 30–60 min. The latex was sieved on reception at the laboratory, to remove any particles of bark, and twenty ultracentrifuge tubes were filled with it. Ten of these were centrifuged as soon as possible in Rotor no. 21 of a Spinco Model L ultracentrifuge. A speed of 20 000 rev./min. ($g_{max.}$ 53 620) was maintained for 40 min.; acceleration and deceleration time together totalled a further 40 min. The refrigeration system of the centrifuge was operated during the run but the latex and rotor were not pre-chilled. The second batch of ten tubes was kept in the refrigerator and centrifuged in a similar manner after the first batch. In this second run the temperature of both latex and rotor was somewhat lower than in the first.

C-serum was collected from the tubes by piercing and draining. It was pooled, filtered and freeze-dried. The bottom fraction was removed after cutting up each tube and lightly washing the surface of the layer with water. Pooled bottom fractions were slurried with water and the slurry was freeze-dried. The freeze-dried materials were sealed under high vacuum and despatched to this laboratory, where they were stored at $-25°$ until required.

METHODS

Elementary analysis. Carbon, hydrogen and ash determinations were made by a Pregl-type microcombustion and total nitrogen was determined by the micro-Kjeldahl method with a mercury catalyst. Phosphorus was measured colorimetrically with ammonium molybdate after wet digestion with sulphuric acid and nitric acid and with Metol (*p*-methylaminophenol sulphate) as reducing agent. The sulphur content was obtained by turbidimetric estimation as barium sulphate, after wet digestion in nitric acid–perchloric acid mixture. The use of this method on a micro scale was examined by Dr W. T. Chambers and a reproducibility of $\pm 5\,\mu$g. over the range 10–150 μg. was obtained. Standards used for calibration were mixtures of pure adenylic acid and insulin, of suitable phosphorus/sulphur ratio.

Hydrolysis. Nucleic acid hydrolyses were carried out in N-HCl at 100° for 1 hr. and in 0·3 N-NaOH at 37° for 16 hr. Samples of approximately 5 mg. of solid were sealed in Pyrex tubes of 0·75 ml. capacity and hydrolysed in 0·2 ml. of acid or in 0·5 ml. of alkali.

To hydrolyse the protein component a sample of about 2 mg. of nucleoprotein was sealed under N_2 in a 0·7 ml. thick-walled tube with 0·4 ml. of a 50:50 (v/v) mixture of 57% HI and 95% formic acid. This was heated at 90–100° for 22 hr.

Chromatography. The nucleic acid constituents were chromatographed according to Wyatt (1951) and the amino acid hydrolysates with both butan-2-one–propionic acid–water (15:5:6, by vol.) (see Clayton & Strong, 1954) and phenol–0·1 N-$NaHCO_3$–water (72:19:9, by vol.) in two-dimensional chromatograms with 2:6-lutidine–propan-2-ol–water–diphenylamine (50:20:25:0·66, by vol.) as the second solvent.

An atmosphere of NH_3 and coal gas, in the presence of KCN solution, was maintained in the tank throughout the phenol runs (see Block, Le Strange & Zweig, 1952).

In all cases hydrolysate solutions were applied and measured with an Agla micrometer syringe (Burroughs Wellcome and Co., London).

Ionophoresis. No. 1 filter paper was used in an apparatus similar to that described by Markham (1955). Sodium citrate or sodium formate buffers (0·3 M) at pH 3·3 were employed.

Detection and estimation of hydrolysis products. Chromatograms or electrophoresis diagrams of the hydrolysis

products were photographed according to Markham & Smith (1949). The resulting prints enabled the components absorbing in the 260 mμ region to be located, and these were then eluted into 0·1N-HCl and quantitatively estimated in a Hilger spectrophotometer, by using the extinction data of Markham & Smith (1951), which was correct for a 5% loss of pyrimidine components. Alternatively, the location and semiquantitative estimation of the purine and pyrimidine components could be made directly and automatically by passage of the filter-paper strips through a short-wave ultraviolet scanning photometer (A. I. McMullen, unpublished work). The nucleic acid content of the original sample was calculated from the total weight of the individual components.

Isolation of ribonucleoprotein. To remove low-molecular-weight nucleotides, sugars etc., the latex freeze-dried C-serum was homogenized in an Ato-mix apparatus (M.S.E. Ltd., London) with HCl–sodium dihydrogen citrate buffer at 0° and pH 3·0 (approximately 20 g./100 ml.). After 3 min. the mixture was centrifuged at 6000 *g* for 15 min. at the same temperature and the insoluble residue extracted twice more with the cold buffer. No nucleic acid was removed at this stage. The residue was then dropped into either (*a*) 0·5M- or 2M-NaCl at 5° or (*b*) 2M-NaCl solution at 95–100°, stirred and maintained at this temperature and kept at pH 6–8 by the addition of NaOH, for 30 min. The insoluble material was separated by centrifuging at 13 000 *g* for 20 min. and re-extracted thrice more with the NaCl solution. The combined extracts were filtered to remove a small quantity of aggregated rubber and brought to 66% (v/v) ethanol content. The ribonucleoprotein (RNP), together with some free protein, was precipitated on standing for a short time at 5° and was centrifuged down. This impure product was redissolved and precipitated with ethanol as before. Repeated precipitation with 0·1N-HCl at pH 3·5 gave a material containing nucleic acid associated with protein. Attempts to remove this protein by denaturation with octanol, chloroform and water (Sevag, Lackman & Smolens, 1938) were unsuccessful, as much of the ribonucleic acid (RNA) was carried down with the denatured protein.

Removal of further protein was achieved by extracting the preparation with a 50:50 (w/w) mixture of A.R. phenol and water at pH 7·0, after the manner of Kirby (1956). By this means a product containing 62% of nucleic acid could be prepared. However, by repeated precipitation of the impure RNP with cetyltrimethylammonium bromide (CTAB), products were finally obtained containing in method (*a*) 60–65%, and in method (*b*) 67–72%, of nucleic acid (as determined by the hydrolytic procedure described above). These latter products were homogeneous in the ultracentrifuge.

The use of CTAB as precipitant was adopted after trials with several cationic compounds. For example, an aqueous solution of 2-methyl-5-vinylpyridine polymer formed such a stable complex with the RNP that it was impossible to separate the two by precipitation methods. This complex was insoluble above pH 5·7 and the nucleic acid could not be precipitated below this pH. The use of CTAB had the further advantage of not absorbing appreciably in the short-wave ultraviolet region.

In carrying out the purification procedure with CTAB about 10 mg. of the impure nucleoprotein was dissolved in 20 ml. of water, dilute NaOH being used to maintain the solution at or near pH 7·0. After stirring for 1 hr. at 5° the insoluble residue was rejected. A 2% CTAB solution (3–5 ml.) was then added with stirring and the colloidal precipitate first formed was converted into a floc by excess of CTAB. When this occurred the complex was centrifuged off, resuspended in ice-cold water and immediately recentrifuged for 10–15 sec. at 2000 *g*. (Some nucleic acid may be lost as a colloidal dispersion if washing is prolonged.) The complex was next dissociated by dissolving in M-NaCl at approximately pH 7·0 and the clear solution, after filtration, adjusted to pH 3·0 with HCl. This caused precipitation of the ribonucleoprotein on standing for a few minutes at 5°. The process was repeated at least once, and until no material insoluble in the salt solution remained. Inorganic salts and residual CTAB were finally removed by precipitation with HCl solution at pH 3·5, after which the RNP was washed with 66% aqueous ethanol and then with absolute ethanol, and dried *in vacuo*.

Sedimentation. The ultracentrifuge runs were carried out on a Spinco machine by Mr B. Boon of the Department of Biochemistry, Cambridge. The runs were performed at a maximum *g* of 270 000 and an average temperature of 20–21°. The solvent was 0·2M-NaCl in all cases, and with the nucleoprotein of the serum solids measurements were made at both pH 7·0 and pH 4·0.

Osmotic molecular weight. This was determined at 0° and at pH 4·0 in the presence of 0·2M-NaCl, with the improved toluene osmometer of Adair (Alexander & Johnson, 1949). Membranes suited to the molecular weight were prepared from collodion solution, the volume of the 'thimble' being approximately 0·7 ml. To reduce loss of solute from this small volume by diffusion into the main osmometer buffer, a glass ball was inserted in the capillary tube just above the membrane. Suitable temperature control at 0° was obtained by placing the osmometer in a vacuum flask containing ice, ensuring that the outer buffer level was appreciably below the level of the ice, and installing the whole in a laboratory refrigerator.

RESULTS

Ultraviolet absorption and fluorescent emission photographs of descending chromatogram patterns of the purified ribonucleoprotein, after acid hydrolysis, showed the presence of four components which were identified from their R_F values as guanine, adenine, cytidylic acid and uridylic acid. Confirmation of the presence of these components was obtained by paper electrophoresis. Corresponding data obtained after alkaline hydrolysis of the nucleoprotein showed, as expected, the presence of the four mononucleotides—guanylic acid, adenylic acid, cytidylic acid and uridylic acid. Further confirmation of the identity of the hydrolysis products was obtained by elution of the spots from the chromatograms and electrophoresis patterns, followed by determination of the spectra over the range 220–300 mμ at pH 1·0 and pH 7·0 or 11·0. Alterations in $\lambda_{max.}$ and in $E_{max.}/E_{min.}$ similar to those expected (Beaven, Holiday & Johnson, 1955) were obtained. In some of the hydrolysates the purine components were also precipitated as silver

Table 1. *Base composition of* Hevea *ribonucleic acid*

Samples 2, 4 and 5 were finally purified by the CTAB procedure and sample 3 by the two-phase phenol treatment.

Sample	Temp. of extraction	Nucleic acid content (%)	Base molar ratio (adenine = 1·0)			Purine / Pyrimidine	6-Oxo / 6-Amino
			Guanine	Cytosine	Uracil		
1	5°	43·9	1·43	1·13	0·95	1·17	1·12
2	5°	59·6	1·50	1·32	1·00	1·08	1·08
3	100°	62·1	1·40	1·21	1·00	1·09	1·09
4	100°	72·1	1·41	1·36	0·80	1·12	0·93
5	100°	71·1	1·51	1·38	0·91	1·10	1·02

salts at pH 1·0, redissolved in boiling HCl solution and determined spectroscopically as above.

Table 1 gives the purine and pyrimidine base composition of typical preparations obtained by different purification procedures. Sample 1 was purified by precipitation with aqueous ethanol and with the addition of 0·1 N-HCl to pH 3·5. Samples 2, 4 and 5 were also subjected to precipitation with CTAB, and sample 3 to phenol treatment similar to that of Kirby (1956), without purification by CTAB. The starting material for samples 1–4 was freeze-dried C-serum, and for sample 5 was bottom-fraction solids. The base composition of the products obtained by hot extraction of the starting materials is very similar to that of the products obtained by extraction at 5°, although the percentage of nucleic acid in the latter samples is lower. A further difference, not shown in Table 1, is that not all the nucleic acid was extracted from the original materials by sodium chloride solution at 5°, even when the concentration of sodium chloride was increased to approximately 2 M.

Hydrolysis of the above samples by the mixture of concentrated HI and formic acid, followed by two-dimensional paper chromatographic analysis, enabled the following amino acids to be identified from their R_F values: aspartic acid, glutamic acid, glycine, serine, cystine, threonine, alanine, lysine, histidine, tyrosine, tryptophan, leucine/isoleucine and phenylalanine. The presence of the aromatic amino acids was confirmed by their ultraviolet absorption. The sulphur content of the preparations containing about 70 % of nucleic acid was 0·3 % and the N/P ratio approximately 5·0.

That the polynucleotide is not deoxyribonucleic acid is apparent from its degradation by alkali, and this is confirmed by a negative diphenylamine reaction. The presence of a pentose sugar in the purified nucleoprotein was confirmed by a positive orcinol test.

The average polynucleotide content of the freeze-dried C-serum was approximately 0·1 %, representing 0·007 % of the original normal latex centrifuged serum.

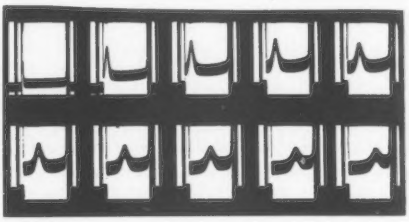


Fig. 1. Sedimentation pattern of *Hevea* ribonucleoprotein in 0·2 M-NaCl at 20° and pH 4·0. (Exposures at 14 min. intervals.) $g_{max.}$, 270 000; rev./min., 59 780.

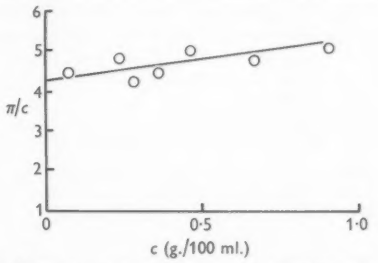


Fig. 2. Osmotic pressure of *Hevea* ribonucleoprotein in 0·2 M-NaCl at 0° and pH 4·0 (mol.wt. 39 400). Ordinate: the 'reduced pressure'.

Incubation of the pure nucleoprotein with 8 M-urea at 37° for several days caused no change in its composition (see Elson, 1959).

Centrifuging of the C-serum solution for 8 hr. at 0° and 25 000 *g* gave a sediment containing about 2 % of the total polynucleotide content.

Molecular weight. Sedimentation data for the purified nucleoprotein (Fig. 1) showed the presence of a single component. Sedimentation coefficients (S) for different preparations varied only from 3·55 to 3·82 s over the pH range 4·0–7·0. Determinations made on a single purified product from freeze-dried bottom fraction gave a similar value of S (3·97).

No diffusion or molar-density data are yet available for this nucleoprotein, but the average value of the sedimentation coefficients at pH 7·0 can be used to obtain a rough estimate of the molecular weight by employing a diffusion coefficient of $D_{20} = 6{\cdot}5 \times 10^{-7}$ and a specific molar volume of $V_p = 0{\cdot}58$. These values were obtained for yeast nucleic acid by Watanabe & Iso (1950) and give a molecular weight of 34 700 in the present instance. This result is almost certainly appreciably lower than the correct value, since the *Hevea* nucleoprotein, containing 30 % of protein, must have a smaller diffusion coefficient and a larger specific volume. Osmotic determinations of mol.wt. (Fig. 2) gave a value of 39 400.

DISCUSSION

Although the analytical results given above provide strong evidence that at least a part of the nucleic acid in *Hevea brasiliensis* latex is present as a soluble ribonucleoprotein, it is difficult to assess the extent to which the different isolation and purification procedures degrade or otherwise affect the native nucleoprotein.

Table 1 shows that the base composition of the purified products is not greatly altered by the method of isolation. The guanine/adenine ratio is considerably higher than in yeast RNA (Magasanik, 1955) or some plant-leaf RNA (Thomas & Sherratt, 1956; Lengyel & Ochoa, 1958) and plant-virus RNA (Dorner & Knight, 1953). It is similar, however, to the ratio found by Cooper & Loring (1957) in plant chloroplasts, to that found by Eggman, Singer & Wildman (1953) in tobacco-leaf cytoplasm, and to that in the cytoplasm of some rat cells (Magasanik, 1955). The cytosine/uracil ratios of Table 1 are greater than unity, in contrast with a ratio of less than unity for yeast RNA.

Since the nucleic acid content of the *Hevea* RNA preparation of molecular weight 39 400 was 67 %, it follows that the polynucleotide of the nucleoprotein has a maximum molecular weight of approximately 26 400, representing about 82 nucleotide units. Similarly, the molecular weight of the amino acid component (if protein) cannot exceed 13 000.

Clearly, also, the protein component is not a ribonuclease similar to that described by Elson (1959), since no hydrolysis of polynucleotide occurs on incubation with urea.

An interesting feature of the present results is the relatively high percentage of nucleic acid in the samples purified by the CTAB procedure. Even so, these samples contained 30 % by weight of non-nucleotide component, which is evidently very firmly bound to the polynucleotide to give a product showing only one peak in the ultracentrifuge. It has not yet been possible to determine quantitatively the amino acid content of the hydrolysed products, but, assuming that all the non-nucleotide portion of the purified samples is composed of amino acid residues with an average molecular weight of 118, the ratio of mono-nucleotide to amino acid residues in these samples can be calculated readily from the nucleotide base compositions in Table 1. This ratio is approximately 1·0 for the preparations with the highest nucleic acid content, suggesting the possibility that removal of protein by methods which do not affect the polynucleotide composition results in a 'core' of adhering amino acid residues where each residue is associated with one nucleotide. The mechanism of this adherence has not been investigated in this work, but a theory such as that postulated by Dounce (1952) for amino acid and polynucleotide interaction, or the possibility of polypeptide–polynucleotide combination by a similar mechanism where only the basic residues react (probably enhanced in this case by van der Waals interaction), could be put forward. Carboxyl groups in the former instance or surplus phosphate and carboxyl groups in the latter instance would still be capable of reacting with CTAB to give the insoluble complex described above.

SUMMARY

1. The isolation and characterization of a purified ribonucleoprotein component of *Hevea* rubber latex is described.

2. A method of final purification employing a cationic detergent has been developed, which gives a product of approximately 70 % nucleic acid content.

3. The composition of the polynucleotide portion of the purified product is similar to that of several other plant nucleic acids so far reported in cytoplasm.

4. The molecular weight of the product is approximately 40 000; the protein part of the molecule has a maximum molecular weight of 13 000.

5. The ratio of the number of amino acid residues to the number of nucleotide residues in the purified nucleoprotein is discussed.

I wish to thank Mr G. F. J. Moir and the staff of the Rubber Research Institute of Malaya for their co-operation in the supply of all latex materials, Dr E. G. Cockbain for helpful discussions and Drs Roy Markham, F.R.S., and G. S. Adair, F.R.S., for suggestions about techniques. My thanks are also due to Mrs I. C. Barnes for technical assistance in all aspects of the work, to Mr G. Higgins for determinations of some of the spectra and to Dr W. T. Chambers for most of the elementary analyses.

This work forms part of a programme of fundamental research undertaken by the Board of the British Rubber Producers' Research Association.

REFERENCES

Alexander, A. E. & Johnson, P. (1949). *Colloid Science*, 1st ed., vol. 1, p. 164. Oxford: Clarendon Press.

Beaven, G. H., Holiday, E. R. & Johnson, E. A. (1955). *The Nucleic Acids*: vol. 1, p. 493. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.

Block, R. J., Le Strange, R. & Zweig, G. (1952). *Paper Chromatography; A Laboratory Manual*. New York: Academic Press Inc.

Clayton, R. A. & Strong, F. M. (1954). *Analyt. Chem.* **26**, 1362.

Cook, A. S. & Sekhar, B. C. (1953). *J. Rubb. Res. Inst. Malaya*, **14**, 163.

Cooper, W. D. & Loring, H. S. (1957). *J. biol. Chem.* **228**, 813.

Dorner, R. W. & Knight, C. A. (1953). *J. biol. Chem.* **205**, 959.

Dounce, A. L. (1952). *Enzymologia*, **15**, 251.

Eggman, L., Singer, S. T. & Wildman, S. G. (1953). *J. biol. Chem.* **205**, 969.

Elson, D. (1959). *Trans. Faraday Soc.* **55**, 496.

Homans, L. N. S. & van Gils, G. E. (1948). *Proc. 2nd Rubb. Tech. Conf.*, p. 292.

Kirby, K. S. (1956). *Biochem. J.* **64**, 405.

Lengyel, P. & Ochoa, S. (1958). *Biochim. biophys. Acta*, **28**, 200.

Magasanik, B. (1955). *The Nucleic Acids*, vol. 1, p. 403. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.

Markham, R. (1955). *Modern Methods of Plant Analysis*, vol. 4, p. 246. Berlin: Springer-Verlag.

Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.

Markham, R. & Smith, J. D. (1951). *Biochem. J.* **49**, 401.

Riches, J. P. & Gooding, E. G. B. (1952). *New Phytol.* **51**, 1.

Ruinen, J. & de Haan-Homans, L. N. S. (1950). *Arch. Rubbercult. Ned.-Ind.* **27**, 243.

Sevag, M. G., Lackman, D. B. & Smolens, J. (1938). *J. biol. Chem.* **124**, 425.

Thomas, A. J. & Sherratt, H. S. A. (1956). *Biochem. J.* **62**, 1.

Watanabe, I. & Iso, K. (1950). *J. Amer. chem. Soc.* **72**, 4836.

Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

A Study of Protein-Binding in the Metabolism of Vitamin B_{12}

By MARGARET E. GREGORY and E. S. HOLDSWORTH*

National Institute for Research in Dairying, University of Reading, Shinfield, Berks

(*Received* 3 *November* 1958)

The mechanism by which intrinsic factor from normal human gastric juice promotes the absorption of cyanocobalamin (vitamin B_{12}) in patients with pernicious anaemia is still obscure. In particular there is no agreement about the part played by that substance in the juice which combines with cyanocobalamin and makes it non-diffusible. Substances that combine with cyanocobalamin have been reported in other body fluids and tissues. Rosenthal & Sarett (1952) and Wolff, Karlin & Royer (1952) have shown that human serum contains its vitamin B_{12} in bound form and that the serum will further bind limited amounts of added cyanocobalamin. With an ultrafiltration technique we showed that milk of several different species contains protein-bound vitamin B_{12} and we measured the quantity of additional vitamin that the milks would 'bind' (Gregory & Holdsworth, 1955*a*).

Confusion has arisen because different authors use the term 'free vitamin B_{12}' to mean quite different entities (cf. Heathcote & Mooney, 1958). It seems to us that it can be resolved only if authors would restrict the term to vitamin B_{12} itself, i.e. either cyano- or hydroxo-cobalamin, and to use the term 'available vitamin B_{12}' when implying that the test material can provide a substance that will support the growth of an assay organism.

Even then it is necessary to define carefully the conditions of test since we have shown that the available vitamin B_{12} measured depends on the concentration of the test substance and on the organism and method of assay (Gregory & Holdsworth, 1957). Unfortunately there is no completely satisfactory general method for the measurement of 'free vitamin B_{12}'. Gregory & Holdsworth (1957) concluded that the ultrafiltration method was the most reliable technique for measuring the binding capacity of a substance for additional cyanocobalamin. But it must be remembered that this technique will differentiate only those bound forms too large to pass through the membrane. Certain peptide conjugates of the vitamin will pass through Visking tubing although they are obviously bound forms of vitamin B_{12}. Butan-1-ol extracts free vitamin B_{12} from test material saturated with ammonium sulphate (Ellis, Petrow & Snook, 1949) and also certain small peptide conjugates.

In the experiments reported in this paper we have used the ultrafiltration method to determine whether tissues contain any ultrafiltrable vitamin

* Present address: Department of Biochemistry, University of Adelaide.

B_{12} activity, and to measure their ability to combine with added cyanocobalamin. Microbiological, physicochemical and immunological tests on the bound forms are reported which show that the bound forms of vitamin B_{12} from the same tissues of different species and sometimes from different tissues of the same animal are not identical.

The high cyanocobalamin-binding activity of chick serum enabled us to isolate a pure cyanocobalamin–protein complex from it and to compare it with similar complexes isolated from pig pylorus (Andresen & Skouby, 1956) and from sow's milk (Gregory & Holdsworth, 1955*a*, *b*).

EXPERIMENTAL AND RESULTS

Materials

Bendogen is a commercial preparation of intrinsic factor from pig pyloric mucosa (G.E.A., Copenhagen). The pure cyanocobalamin–protein complex from Bendogen was a gift from Dr J. G. Andresen (G.E.A., Copenhagen). Its preparation was described by Andresen & Skouby (1956). The vitamin B_{12}–polypeptide was prepared from liver and was a gift from Dr H. G. Wijmenga (Organon Laboratories, Oss, Holland). The preparation of the cyanocobalamin-binding factor from sow's whey was described by Gregory & Holdsworth (1953).

Samples of serum were obtained from venous blood of the cow, goat, pig, rabbit and man. The chick and rat sera were obtained by exsanguination of freshly killed animals.

Extracts of parts of the intestinal tract were prepared as follows: whole pig or rat stomachs, a sheep abomasum or chick proventriculi were cut open, rinsed briefly with water to remove adhering food, and homogenized in a Waring Blendor with acid NaCl soln. (0·9% NaCl in 0·05 N-HCl). The homogenate was centrifuged and the solid residue re-extracted twice with further portions of acid NaCl soln. The combined supernatant liquids were dialysed against running tap water for 24 hr., filtered and the clear filtrate was freeze-dried. Human gastric juice with intrinsic-factor activity was obtained from Dr D. L. Mollin (Postgraduate Medical School, London).

Livers were collected from six freshly killed vitamin B_{12}-deficient chicks prepared according to the method of Coates, Harrison & Kon (1951). The bulked livers (24 g.) were homogenized with water and made to a total volume of 48 ml. Livers from normal chicks were treated similarly.

[^{60}Co]Cyanocobalamin with specific activity 1 μc/mg. was kindly supplied by Glaxo Laboratories Ltd., Greenford, Middlesex.

Measurement of bound forms of vitamin B_{12} in animal tissues, organs and body fluids

The natural vitamin B_{12} content of tissues, organs and body fluids of some representative species was measured in tube assays with *Lactobacillus leichmannii* A.T.C.C. 4797 after digestion of the sample with papain as described by Gregory (1954). In our experience treatment with papain is necessary to make the vitamin in these materials fully available to the test organism. The capacity of these tissues and organs to combine with additional cyanocobalamin was measured by an ultrafiltration technique (Gregory & Holdsworth, 1955*a*, 1957). The results are shown in Table 1.

In general, serum can bind additionally about as much vitamin B_{12} as it originally contains. The one

Table 1. *Vitamin B_{12} content and vitamin B_{12}-binding activity of serum and of tissues of some animals*

Test organism: *L. leichmannii* A.T.C.C. 4797; assay conditions were as described in the text. Values for serum are given as μmg./ml.; those for tissues are as μmg./g. wet wt. unless otherwise indicated.

	Vitamin B_{12} content	Binding capacity for additional cyanocobalamin
Serum		
Cow	2·3	3·5
Goat	3·3	4·4
Chicken	4·3	1 100·0
Man	1·2	2·0
Pig	0·4	4·5
Rabbit	46·0	17·0
Rat	0·8	3·8
Gastrointestinal tract		
Human gastric juice*	0	68
Chicken proventriculus	140	1 700
Chicken duodenum	0	0
Stomach of a newborn pig	0	200
Pig-pylorus extract (Bendogen)†	5	220 000
Rat stomach	70	700
Sheep abomasum (cardiac half)	0	0
Sheep abomasum (pyloric half)	0	100
Liver		
Chicken (normal)	86	117
Chicken (B_{12}-deficient)	13	127
Pig (14 days-old)	23	8

* μmg./ml. † μmg./g. of freeze-dried extract.

exception is chick serum, which has a natural vitamin B_{12} content similar to that of the other species tested, but a binding capacity for additional cyanocobalamin some 300 times this amount. The rabbit serum had the highest vitamin B_{12} content. Autoclaving the serum samples with alkali destroyed all their growth activity for *L. leichmannii*, showing that deoxyribosides were not interfering with the measurement of vitamin B_{12} by this organism.

Material with high cyanocobalamin-binding activity was also obtained by extracting certain portions of the gastrointestinal tract of different species. It can be seen from the table that Bendogen prepared from pig pylorus and having intrinsic-factor activity had a high vitamin B_{12}-binding capacity. The vitamin B_{12}-binding factor can even be detected in the stomach of the newborn pig.

The proventriculus (i.e. the glandular stomach) of the chick also has considerable binding activity whereas none could be detected in the duodenum. The pyloric and cardiac halves of the abomasum of a sheep were tested separately and the vitamin B_{12}-binding activity was found in the pyloric half only. Equal weights of liver homogenates from normal and vitamin B_{12}-deficient chicks can combine with about the same amount of additional cyanocobalamin, although on the same weight basis the normal livers have the higher natural vitamin B_{12} content.

The vitamin B_{12} present in the chick-, rat- and pig-liver homogenates and in all serum samples tested was not ultrafiltrable. The ultrafiltration method thus confirms that in the liver and serum of the species tested there is no free vitamin B_{12}.

When human gastric juice was mixed with human serum, the total binding activity, measured by the ultrafiltration method, did not exceed the sum of the individual activities of the serum and gastric juice. Thus we were not able to confirm the work of Miller (1957), who reported that gastric juice potentiated the binding capacity of the serum. We applied Miller's charcoal method for measuring binding activity and found that it gave much lower results than the ultrafiltration method, which we attributed to the fact that charcoal could adsorb bound forms of vitamin B_{12} besides the free vitamin, and confirmed this supposition in experiments with Bendogen combined with [^{60}Co]cyanocobalamin.

Examination of the bound forms by electrophoresis

[^{60}Co]Cyanocobalamin was added in slight excess over the binding capacity to extracts of pig pylorus (Bendogen), rat stomach and chick proventriculus prepared as described under Materials, and to chick serum, human gastric juice and human serum. Suitable amounts were subjected to paper electrophoresis at various pH values. The position of the proteins was revealed by staining with bromophenol blue and that of cyanocobalamin by radioautography as described previously (Gregory & Holdsworth, 1955*a*).

As we had previously observed with milk, each tissue gave only one zone on paper electrophoresis that was responsible for the cyanocobalamin-binding activity. Normal human serum was an exception and showed a small second peak of activity in the α_1-globulin region. Fig. 1 shows the

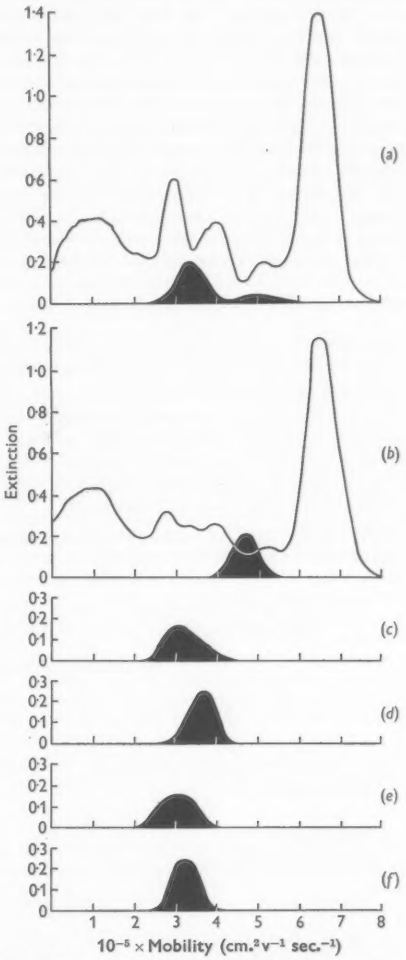


Fig. 1. Electrophoretic patterns of serum and extracts of intestinal tract. Electrophoresis in sodium veronal buffer, pH 8·6. The filled-in curves represent added [^{60}Co]cyanocobalamin measured as the extinction of the radioautographs. Other curves represent protein distribution. Ordinates are arbitrary units of extinction. (*a*) Normal human serum; (*b*) chick serum; (*c*) chick-proventriculus extract; (*d*) rat-stomach extract; (*e*) human gastric juice; (*f*) pig-pylorus extract.

mobilities of some of the complexes at pH 8·6. Under these conditions free cyanocobalamin had zero mobility. It can be seen that the binding component in serum is not one of the well-characterized serum proteins. In extracts of the stomach of the chick, rat and pig, and in human gastric juice, the specific proteins have a mobility of $-3{\cdot}0$ to $-3{\cdot}5 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ at pH 8·6 in 0·05M-veronal buffer. Although the mobilities were similar at pH 8·6 it was possible to distinguish between some of these materials by examination at other pH values. At pH 6·4 the bound form prepared from chick liver had a higher mobility than that prepared from chick proventriculus. Near the isoelectric point, pH 4·0–5·0, it was possible to show differences in mobility between the bound vitamin B_{12} in pig-pylorus extract, sow's milk and rat-stomach extract. Chick serum differed from the other materials tested in that the bound form had a high mobility ($-4{\cdot}9 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$) close to that of α_1-globulin at pH 8·6.

Examination in plate assays of the growth activities of bound forms of vitamin B_{12} for Escherichia coli *and* Lactobacillus leichmannii

In a previous paper we have shown that cyanocobalamin combined with Bendogen had growth activity for *E. coli* when assayed unheated on agar plates (Gregory & Holdsworth, 1957). This led us to test other bound forms of the vitamin.

The *E. coli* plates were prepared as described by Gregory & Holdsworth (1957). The test solutions (0·01 ml.) were applied to the plates on 6 mm. disks of Whatman no. 4 filter paper. 2:3:5-Triphenyltetrazolium chloride was added to facilitate measurement of the zones of growth (Ford & Holdsworth, 1953). The vitamin B_{12} activity of the test preparations was calculated both from the measurement of the diameters of the growth zones and by measurement of the amount of red formazan pigment eluted from the growth zones with tetrahydrofuran.

The *L. leichmannii* plate-assay method was essentially the same as the *E. coli* method, except that the medium of Skeggs, Nepple, Valentik, Huff & Wright (1950) was used at pH 6·8 with 2% of agar and 0·1 g. of ascorbic acid (added under aseptic conditions)/150 ml. of medium. 2:3:5-Triphenyltetrazolium chloride was not added. Each plate was inoculated with 4 ml. of a 24 hr. culture of *L. leichmannii* grown in the basal medium supplemented with 0·01 μmg. of cyanocobalamin/ml. The cyanocobalamin standard was the same as that used for the *E. coli* plates. Incubation was for 18 hr. at 37°. The solutions to be tested contained no ultrafiltrable vitamin B_{12} activity. The vitamin B_{12} content of the complexes was determined by the papain-digestion method in tube assays with *L. leichmannii* as the test organism, as described by Gregory (1954).

The growth activities of the different cyanocobalamin complexes tested at various levels of cyanocobalamin content are shown in Table 2. It can be seen that there was no correlation between the cyanocobalamin content of the various bound forms and their growth activities for both test organisms. For example, the human-milk and chick-serum complexes were completely inactive at all levels tested, whereas the preparations from sow's milk, chick proventriculus, rat stomach and pig pylorus all had some growth activity, which

Table 2. *Activity of bound forms of cyanocobalamin (expressed as a percentage of that of free cyanocobalamin) for* Lactobacillus leichmannii *and* Escherichia coli *in plate assays*

Test preparation (0·01 ml.) was applied to 6 mm. disks.

Source of bound cyanocobalamin	Amount of cyanocobalamin in complex (μg./ml.)	Activity for *L. leichmannii* calc. from growth-zone diameter	Activity for *E. coli*	
			Calc. from growth-zone diameter	Calc. from colour of zone
Sow's milk	1·1	35	40	36
	0·11	44	45	91
Human milk	0·13	1	0	0
Human gastric juice	0·09	106	76	*
	0·009	111	111	*
Chicken serum	1·0	0	0	0
	0·1	0	0	0
Chicken proventriculus	1·0	8	9	10
	0·1	12	19	10
Rat stomach	0·7	36	48	83
	0·07	49	77	165
Liver (polypeptide–vitamin B_{12} complex)	0·3	87	123	93
	0·03	100	103	93
Bendogen (100 mg.) + 20 μg. of vitamin B_{12}	1·5	0	0	0
	0·15	2	10	11
	0·015	28	63	67

* Not measured.

increased as the test solutions were diluted. Cyanocobalamin added to undiluted human gastric juice was fully available to *L. leichmannii* and 76 % available to *E. coli*. On further tenfold dilution the vitamin was fully available to both organisms. The liver vitamin B_{12}–polypeptide complex was as active as the free vitamin at all levels tested.

Effect of enzymes on bound cyanocobalamin and on binding activity

In order to study whether binding activity could be altered by treatment with enzymes, portions (1 mg.) of the sow's-whey binding factor were digested at 55° for 3 hr. with the following enzymes (preparations from The Armour Laboratories unless otherwise indicated): in buffer of the appropriate pH, 10 mg. of papain (British Drug Houses Ltd.) activated with cyanide, 2 mg. of crystalline trypsin, 2 mg. of chymotrypsin, 2 mg. of carboxypeptidase or 2 mg. of crystalline pepsin. Half of the enzyme was added at the beginning of the experiment and the remainder halfway through the incubation period. After digestion, 0·1 μg. of cyanocobalamin (slightly more than sufficient to saturate the binding activity) was added to each of the digests, which were then brought to a volume of 10 ml. and a portion of each was removed for measurement of available vitamin B_{12}. The remainder of each digest was ultrafiltered and the vitamin B_{12} activity of the ultrafiltrates measured. Both the digest and ultrafiltrate were assayed without further treatment other than dilution with water. The test organism was *L. leichmannii*. The ability of these same enzymes to release cyanocobalamin from the combination with the binding factor was studied simultaneously in the same way. In this part of the experiment, the cyanocobalamin (0·1 μg.) was added to 1 mg. of the binding factor before treatment with the enzymes as described above.

The results of this experiment are shown in Table 3. When 0·1 μg. of cyanocobalamin was added to the untreated binding factor, only 0·01 μg. appeared in the ultrafiltrate. Digestion of the binding factor with papain, trypsin or pepsin reduced its ability to combine with added cyanocobalamin and from 50 to 60 % of the added vitamin appeared in the ultrafiltrate (see column 4 in Table 3). After digestion with chymotrypsin and carboxypeptidase the binding activity of the sow's-whey factor was also reduced, but to a lesser extent than with the other enzymes. However, when the binding factor was saturated with cyanocobalamin before treatment with the enzymes it was more resistant to their attack. Column 2 in Table 3 shows that there was no increase in the ultrafiltrable cyanocobalamin resulting from enzyme treatments of the bound vitamin. Therefore the enzymes were not able to release free cyanocobalamin from the binding protein, although they were able to increase the microbiological availability of the vitamin in the complex.

In the presence of untreated sow's-whey binding factor, added cyanocobalamin was only 60 % available, whereas cyanocobalamin added to any of the enzyme digests of the binding factor was fully available to *L. leichmannii*. When the enzymes acted on the binding factor saturated with cyanocobalamin, only papain digestion made the vitamin fully available to *L. leichmannii*.

Immunological tests

Cyanocobalamin–protein complex (40 mg.) from pig pylorus adsorbed on alumina (Proom, 1943) was injected intramuscularly into the leg of a 3 kg. rabbit. Blood serum obtained 2 weeks later contained precipitating antibodies, 5 ml. of antiserum being sufficient to precipitate in 3 hr. at room temperature 85 % of a [^{60}Co]cyanocobalamin–Bendogen complex containing 5 mg. of Bendogen and 1 μg. of cyanocobalamin. The amount of precipitate, however, appeared to be independent of the presence or absence of the cyanocobalamin.

Rough quantitative tests (ring precipitin) with dilutions of antigen were performed, by using various binding substances which had been saturated with radioactive cyanocobalamin and freed from excess by prolonged dialysis. When quantities of the different materials with equal binding capacities were tested against the antiserum, pig-pylorus and sow's-milk antigens gave closely similar reactions. Other complexes (from chick serum, chick proventriculus, rat stomach and human gastric juice) showed cross-reactions, but only at much higher concentrations.

Table 3. *Effect of enzyme digestion on the binding of* 0·1 μg. *of cyanocobalamin by* 1 *mg. of the factor from sow's whey and on the microbiological availability of cyanocobalamin combined with the sow's-whey factor*

Test organism: *L. leichmannii*. Results are the means of two assays.

	Cyanocobalamin (0·1 μg.) added before enzyme digestion		Cyanocobalamin (0·1 μg.) added after enzyme digestion	
	Available cyanocobalamin (μg.)	Ultrafiltrable cyanocobalamin (μg.)	Available cyanocobalamin (μg.)	Ultrafiltrable cyanocobalamin (μg.)
Control (no enzyme)	0·06	0·01	0·06	0·01
Papain	0·11	0·01	0·11	0·05
Trypsin	0·09	0·01	0·11	0·06
Chymotrypsin	0·07	0·01	0·10	0·04
Carboxypeptidase	0·07	0·01	0·11	0·03
Pepsin	0·07	0·01	0·10	0·06

Isolation of a cyanocobalamin–protein complex from serum

A concentrate of the binding substance from chick serum was prepared by adding 1 l. of 11 N-HCl to 5 l. of serum at 4° and centrifuging off the precipitated proteins and immediately dialysing the supernatant fluid against running tap water to remove acid. The volume remaining after dialysis was reduced by freeze-drying to approx. 600 ml.; 1 ml. of this concentrate combined with 6 μg. of cyanocobalamin.

A volume (300 ml.) of the concentrate was mixed with a solution containing 2·0 mg. of [^{60}Co]cyanocobalamin and cooled to 4°. Saturated ammonium sulphate solution at the same temperature was added, with stirring, to 33% saturation. The precipitate was removed by centrifuging and washed with 33% saturated ammonium sulphate solution. Since no radioactivity was detected in the precipitate, it was rejected. A similar precipitate obtained at 50% saturation had only slight radioactivity and was also discarded. Proteins remaining in solution were precipitated by adding solid ammonium sulphate to saturation, again at 4°. The precipitate (containing the radioactivity) was suspended in water and dialysed for 6 hr. against running tap water. The solution (approx. 60 ml.) was refractionated with ammonium sulphate and 66–100% saturation yielded the crude cyanocobalamin complex. After dialysis against distilled water the protein solution (50 ml.) was made 0·025 M with sodium acetate buffer (pH 4·8), and cooled to 1° and propan-2-ol at −12° was added dropwise with stirring. The temperature was gradually lowered to −12° during the fractionation. The various fractions obtained were tested for the presence of cyanocobalamin by dissolving in water and measuring the radioactivity (cf. Gregory & Holdsworth, 1955*a*). About 90% of the cyanocobalamin protein was precipitated in the fraction containing 35–45% (v/v) of propan-2-ol, and this material, dissolved in 20 ml. of water, was refractionated by similar precipitation with propan-2-ol, at pH 4·0.

The resulting pink protein was dissolved in water and dialysed against changes of distilled water. The solution was cooled to 1° and fractionated with 2% (w/v) acetic acid in propan-2-ol, when the pink material precipitated at a concentration of between 10 and 15% of the acid alcohol. Alternate precipitations at pH 4·8 and with acid propan-2-ol were continued until the ratio of the extinction coefficient at 278 mμ (due mainly to protein) and at 362 mμ (due to cyanocobalamin) reached a steady value. After dialysis against distilled water the complex was obtained as a pink powder by freeze-drying the solution (yield: 24 mg.).

The material migrated as a single pink band, with a mobility close to that of α_1-globulin, on paper electrophoresis at pH 8·6. It differed from α_1-globulin in its solubility properties and did not stain on paper with bromophenol blue. In the ultracentrifuge it sedimented as a single symmetrical peak which showed no sign of resolving in 72 min. at 60 000 rev./min. in the Spinco analytical ultracentrifuge. The $S_{20,w}$ for a 0·5% solution was 3·95*s*. As far as could be ascertained the chemical properties resembled those of the two complexes examined previously, one from sow's milk (Gregory & Holdsworth, 1955*b*) and one from pig pylorus (Holdsworth & Ottesen, 1955).

The pink protein contained 15 μg. of vitamin B_{12}/mg., calculated from the absorption at 362 mμ (Gregory & Holdsworth, 1955*b*), 8·9% of total nitrogen (micro-Kjeldahl) and, after hydrolysis for 4 hr. with 2 N-H_2SO_4, 27% of reducing sugars were found by the method of King & Wootton (1956), and 4·5% of methylpentose (probably fucose) by the method of Dische & Shettles (1948). Thus the complex is a glyco- or muco-protein.

DISCUSSION

Pitney, Beard & Van Loon (1954), Ostrowski, Skarżyński & Zak (1954) and Heinrich & Erdmann-Oehlecker (1956) have separated serum proteins by paper electrophoresis and estimated by microbiological methods the vitamin B_{12} present in the major protein fractions of the serum. Each group concluded that the vitamin B_{12} was associated mainly with the α_1- and α_2-globulin. This distribution between different fractions can be explained if the vitamin was combined with a minor component whose properties overlap those of the main serum proteins, and since the papers were cut into these main fractions the vitamin activity would be spread over a number of them. The vitamin B_{12} content and the binding activity of human serum are known to increase in certain diseases such as leukaemia (Heinrich & Erdmann-Oehlecker, 1956). This increase may be associated with the second (minor) binding component of normal human serum since we found that this component was present in a sample of chronic myeloid leukaemic serum to a much greater extent than in normal serum. As shown in Fig. 1, the material in chick serum that combines with additional cyanocobalamin had a mobility between that of α_1- and α_2-globulin. Furthermore, the binding protein constituted less than 0·1% of the total protein of serum and could not have been detected by the usual staining procedures used in paper electrophoresis. With chick serum we have added cyanocobalamin to saturate the binding material and isolated the glycoprotein–vitamin complex, which differed from the α_1- and α_2-globulin in many of its properties. Thus the binding capacity of serum appears to us to be due to a minor specific glycoprotein and not to any of the main protein components.

The bound forms prepared by adding cyanocobalamin to sow's milk, chick serum and pig-pylorus extract have been obtained in a state approaching purity and the complexes have similar chemical and physical properties. The pig-pyloric complex was obtained in purest form and hence was more fully investigated (Gregory, Holdsworth & Ottesen, 1957; Holdsworth & Ottesen, 1955). More recently the binding substance in Bendogen

has been isolated by chromatography on ion-exchange cellulose columns and shown to consist of two binding materials, only one of which had intrinsic-factor activity (Holdsworth, 1957).

The bound forms of cyanocobalamin differ in their microbiological activity when measured by growth of *L. leichmannii* or *E. coli*. The availability of these complexes to both organisms increased in the following order according to the nature of the binding factor: human milk and chick serum, pig stomach (Bendogen), chick proventriculus, sow's milk, rat serum and human gastric juice. The vitamin B_{12}–polypeptide complex from liver was more available than any of the other bound forms, indicating either that the free vitamin is much more readily released from the peptide moiety or that *L. leichmannii* and *E. coli* can utilize the complex as easily as the free vitamin.

Certain animals lack the ability to absorb cyanocobalamin bound to foreign binding proteins, e.g. the chick will readily absorb the vitamin bound to its own binding factors but has difficulty in absorbing cyanocobalamin bound to the sow's-milk binding factor (Coates *et al.* 1955). This finding also suggests that the digestive enzymes of the test animal have little action on the foreign complex otherwise the vitamin would be released and become available. The experiments *in vitro* reported in this paper confirm that the bound vitamin is little affected by several isolated enzyme systems. The immunological work demonstrates a difference between the binding factors of different species.

A substance that combines with cyanocobalamin is not necessarily intrinsic factor, but it seems likely that intrinsic factor combines with cyanocobalamin. Bishop, Toporek, Nelson & Bethell (1955) used the urinary excretion of ^{60}Co as a test for absorption of the labelled vitamin in gastrectomized patients. ^{60}Co appeared in the urine only when the labelled vitamin was actually combined with intrinsic factor. The intrinsic-factor preparation from Bendogen, described by Holdsworth (1957), was active (0·5 mg. with 0·5 μg. of radioactive cyanocobalamin) in absorption (Schilling) tests in patients with pernicious anaemia. This preparation would combine with 15 μg. of cyanocobalamin/mg. If this material can be accepted as a pure substance then there seems no doubt that intrinsic factor combines with vitamin B_{12}. With washed-out loops of small intestine in the surviving rat we were able to show that the bound vitamin B_{12}, formed when the vitamin is added to rat-stomach extract, is absorbed two to three times better than the free vitamin (Holdsworth & Coates, 1956). Similarly, the uptake of the vitamin by surviving mucosal cells was increased by rat-stomach extract (Coates & Holdsworth, 1958). Even if the binding substance in rat stomach extract is not the rat 'intrinsic factor', the bound form was absorbed in greater amount than the free vitamin. If a foreign binding substance was tested in the rat, absorption was depressed below that of the free vitamin (Holdsworth & Coates, 1956), an observation which supports the idea of a species specificity of the binding proteins.

SUMMARY

1. The natural vitamin B_{12} content and the binding capacity for cyanocobalamin of a number of tissues and body fluids of several animal species have been examined. As judged by electrophoresis, each material contained a specific cyanocobalamin-binding glycoprotein.

2. The cyanocobalamin–protein complex obtained by adding the vitamin to chick serum has been isolated. Its properties were similar to those of the other two complexes (from milk and pig pylorus) we have studied.

3. Although similar in some chemical and physical properties, the complexes from different sources had different availabilities for micro-organisms and for animals and differed immunologically.

4. The evidence for specific binding glycoproteins playing an important role in the absorption and retention of cyanocobalamin in the body is discussed.

We are indebted to Dr M. E. Sharpe for advice and help with the immunological work, and to Mrs Olive Bullock and Mr R. Dawson for valuable technical assistance. It is a pleasure to thank Dr S. K. Kon for his encouragement and helpful discussions.

REFERENCES

Andresen, J. G. & Skouby, A. P. (1956). *Acta med. scand.* **155**, 311.

Bishop, R. C., Toporek, M., Nelson, N. A. & Bethell, F. H. (1955). *J. Lab. clin. Med.* **46**, 796.

Coates, M. E., Gregory, M. E., Harrison, G. F., Henry, K. M., Holdsworth, E. S. & Kon, S. K. (1955). *Proc. Nutr. Soc.* **14**, xiv.

Coates, M. E., Harrison, G. F. & Kon, S. K. (1951). *Analyst*, **76**, 146.

Coates, M. E. & Holdsworth, E. S. (1958). *Biochem. J.* **69**, 20P.

Dische, Z. & Shettles, L. B. (1948). *J. biol. Chem.* **175**, 595.

Ellis, B., Petrow, V. & Snook, G. F. (1949). *J. Pharm., Lond.*, **1**, 60.

Ford, J. E. & Holdsworth, E. S. (1953). *Biochem. J.* **53**, xxii.

Gregory, M. E. (1954). *Brit. J. Nutr.* **8**, 340.

Gregory, M. E. & Holdsworth, E. S. (1953). *Biochem. J.* **55**, 830.

Gregory, M. E. & Holdsworth, E. S. (1955*a*). *Biochem. J* **59**, 329.

Gregory, M. E. & Holdsworth, E. S. (1955*b*). *Biochem. J.* **59**, 335.
Gregory, M. E. & Holdsworth, E. S. (1957). *Biochem. J.* **66**, 456.
Gregory, M. E., Holdsworth, E. S. & Ottesen, M. (1957). *C.R. Lab. Carlsberg, Sér. chim.*, **30**, 147.
Heathcote, J. G. & Mooney, F. S. (1958). *J. Pharm., Lond.*, **10**, 593.
Heinrich, H. C. & Erdmann-Oehlecker, S. (1956). *Clin. Chim. Acta*, **1**, 311.
Holdsworth, E. S. (1957). *Biochem. J.* **66**, 59P.
Holdsworth, E. S. & Coates, M. E. (1956). *Nature, Lond.*, **177**, 701.
Holdsworth, E. S. & Ottesen, M. (1955). *Abstr. 3rd Int. Congr. Biochem., Brussels*, p. 116.
King, E. J. & Wootton, I. D. P. (1956). *Micromethods in Medical Biochemistry*. London: J. and A. Churchill Ltd.
Miller, O. N. (1957). *Arch. Biochem. Biophys.* **39**, 301.
Ostrowski, W., Skarżyński, B. & Zak, Z. (1954). *Bull. Acad. polon. Sci.* **2**, 9.
Pitney, W. R., Beard, M. F. & Van Loon, E. J. (1954). *J. biol. Chem.* **207**, 143.
Proom, H. (1943). *J. Path. Bact.* **55**, 419.
Rosenthal, H. L. & Sarett, H. P. (1952). *J. biol. Chem.* **199**, 433.
Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W. & Wright, L. D. (1950). *J. biol. Chem.* **184**, 211.
Wolff, R., Karlin, R. & Royer, P. (1952). *C.R. Soc. Biol., Paris*, **146**, 1008.

The Alkaloids of Hemlock (*Conium maculatum* L.)

DISTRIBUTION IN RELATION TO THE DEVELOPMENT OF THE FRUIT

By J. W. FAIRBAIRN and S. B. CHALLEN
Department of Pharmacognosy, School of Pharmacy, University of London

(*Received* 8 *September* 1958)

Much of the work published on the formation and role of the alkaloids in the plant has been concerned with the problem of the site of synthesis (whether root or leaf) and the determination of possible precursors by suitable feeding experiments. We have investigated the composition and quantity of the alkaloids of a given plant during a critical stage in its life history and have correlated our findings with physiological age, anatomical changes and histochemical tests. The phase of the development of the flower to the mature fruit was selected for study.

Conium maculatum L. (family Umbelliferae) was chosen because (*a*) there was already evidence that the quantity of alkaloid varied markedly as the fruit developed, (*b*) the alkaloids present were relatively simple ones and few in number and (*c*) the alkaloids are said to be mainly restricted to a special layer of the fruit (the coniine layer), which would give scope for anatomical and histochemical studies. Very few members of the Umbelliferae produce alkaloids, the majority producing volatile oils in clearly delimited tissues of the fruit, known as vittae; in *Conium*, vittae occur in the young fruits but almost disappear during maturation.

During the course of our work a paper on the distribution of alkaloids in *Conium maculatum* was published by Cromwell (1956). While Cromwell's work anticipated some of our techniques, and we have made use of some of those which he published, it has not affected the main body of our work. Some of our findings confirm those of Cromwell but since we have made a more detailed study of a restricted part of the life history of the plant, we can make a more complete report of this aspect.

EXPERIMENTAL

Initially two separate samples of seeds were sown in the School of Pharmacy Medicinal Plants Garden at Myddelton House, Enfield, Middlesex; one sample was derived from plants already growing at Myddelton House and the other was obtained from Chelsea Physic Garden, London. It soon became obvious that these two samples represented two varieties of *C. maculatum* which differed slightly in fruit and leaf morphology. More important still we found that one variety (that from Chelsea) produced little or no methylconiine whereas the other one did. To avoid complication in our work, we decided to use only the Chelsea variety of *C. maculatum*.

Sampling techniques. Careful sampling techniques are essential but previous work on alkaloids in plants has often shown a lack of appreciation of this requisite (Dawson, 1948). As our studies were restricted to the flower and fruit we made a careful study of the distribution of the flowers on the growing plant. The inflorescence is a compound umbel consisting of 10–15 rays, and each ray bears a secondary umbel with 8–18 flowers. It was noticed that all the flowers on a given compound umbel developed at practically the same time but that the stage of development varied from compound umbel to compound umbel on the same plant or even on the same branch. It was therefore decided to use the compound umbel, containing on an average about 150 flowers, as the 'unit'. These compound umbels are first recognized as small greenish masses of

tightly coiled buds which soon developed into a flat head of fully opened white flowers. This stage was easily recognized and it was assumed that pollination took place then; this stage was therefore called week 1. In a normal season it took 7–8 weeks for these pollinated flowers to develop into fully ripened, almost dehiscent, fruits. At the beginning of this period 250 compound umbels at the stage of week 1 were tagged by tying a white card just below the point where the rays arise, and 25 of them were collected between 9 and 10 a.m. (G.M.T.) the same day each week during the developing period. From these weekly samples, containing about 3000–4000 fruits, two groups of 800 fruits were placed in tared stoppered flasks as follows: (*a*) with 50 ml. of 70% ethanol (for quantitative chemical work); (*b*) for oven-dry weight determination. The remainder of the fruits were reserved for qualitative chemical examination and for anatomical studies.

The flasks were then re-weighed and the following information was obtained for each weekly collection: (i) the average fresh weight of one fruit; (ii) the average dry weight of one fruit; (iii) the amount of alkaloids, both total and individual, per fruit; (iv) the percentage of alkaloids on a dry-weight and a fresh-weight basis. For the quantitative chemical work the fruits were allowed to macerate in the ethanol for at least 7 days before assay and allowance was made for the amount of water contained in the 800 fruits added to each 50 ml. of 70% ethanol.

By using this sampling technique we obtained a variety of reference bases for our quantitative data and were able to select the most suitable for our purposes and at the same time compare our results with those of other workers even when they had used less suitable sampling methods.

The following methods for the determination of total alkaloids and of individual alkaloids in the fruits were used.

Measurement of the areas of spots on paper chromatograms. The methods described by Fisher, Parsons & Morrison (1948) and Fisher, Parsons & Holmes (1949) for amino acids was applied to the determination of the hemlock alkaloids. The results were insufficiently accurate for our purposes but the method was found to be valuable for preliminary investigations, especially when dealing with small quantities of plant material.

Spectrophotometric methods. The gravimetric method of Farr & Wright (1904) and the titrimetric method of Janot & Fabre (1929) were considered unsuitable as large quantities of material were required. Accordingly, a number of colour reactions reported for the hemlock alkaloids were investigated with a view to devising suitable spectrophotometric methods. The sodium β-naphthaquinone-4-sulphate method of Euler (1945) for piperidine, the chloranilic acid method of Barreto (1954) for coniine, the α-nitroso-β-naphthol method of Reichard (1905) and the carbon disulphide method of Dilling (1909) were all tried but found to be unsuitable. Cromwell (1956) used two colour reactions, based on reactions reported by earlier workers, and we found his methods the most suitable so far reported. Certain alterations were made and the following is an account of the methods we used, together with reasons for changing those described by Cromwell.

Preliminary extraction of the alkaloids from the fruits. A suitable volume (40 ml.) of the 70% ethanolic extract prepared from the weekly collection of fruits was transferred to an evaporating basin, acidified with 5 ml. of N-H_2SO_4 and evaporated to small volume on a water bath to remove most of the ethanol. The contents of the dish were transferred to a separator, with several rinsings of water and a final one of chloroform. The aqueous solution was extracted with chloroform to remove coloured impurities and the chloroform extracted with 2×5 ml. portions of 0·1 N-H_2SO_4, which were added to the original aqueous solution. The solution was then made alkaline to litmus with 10% NaOH solution and extracted with successive portions of chloroform. The mixed chloroform fractions were shaken with a few drops of concentrated HCl (A.R.) and the chloroform was removed by evaporation on a water bath. The mixed hydrochlorides were dried in an oven at about 90° to remove excess of acid and were dissolved in water to 100 ml. This solution was used for further determinations, each of which was made in duplicate. In no instance did the two results differ by more than 3%. Cromwell recommended micro-steam-distillation for the preliminary extraction of the alkaloids: we found this method inconvenient as it was difficult to prevent overflow of froth into the condenser and at the same time maintain an adequate pressure of steam through the mixture.

Determination of the total alkaloids in the fruit. The following solutions were mixed together in a 50 ml. separator: a suitable volume (0·5–15·0 ml.) of the solution of alkaloidal hydrochlorides already referred to, 1 ml. of phosphate buffer [0·1 M-KH_2PO_4 adjusted to pH 7·0 with 0·1 M-KOH and Universal indicator (B.D.H. Ltd.)], 5 ml. of a saturated solution of bromothymol blue in water. The mixture was then extracted with three 5 ml. quantities of benzene; each extract was successively dried with 0·5 g. of anhydrous Na_2SO_4, transferred to a 20 ml. measuring flask and made up to volume with further benzene which had been washed with the Na_2SO_4 previously used. The extinction of the yellow solution in benzene was then determined, with dry benzene as blank, in 0·5 cm. tubes, at 440 mμ with the Unicam D.G. spectrophotometer. The amount of total alkaloid present was calculated from a calibration curve prepared by using pure coniine hydrochloride.

We found that it was necessary to use a larger amount of bromothymol blue than that stated by Cromwell, as this indicator must be present in excess in order to complete the reaction. Moreover, two or three shakings with benzene are necessary in order completely to extract the coloured complex from the aqueous layer; furthermore the Na_2SO_4 used for drying the benzene extract adsorbs an appreciable amount of the coloured complex, and has therefore to be extracted with benzene and this fraction added to the others.

Determination of γ-coniceine. To a 20 ml. volumetric flask were added, in the following order: a suitable volume (0·5–15·0 ml.) of the solutions of alkaloidal hydrochlorides already referred to, aqueous 1% sodium nitroprusside solution (0·6 ml.), aqueous 10% (w/v) Na_2CO_3 solution (0·4 ml.). The mixture was made up to volume with water and the red colour produced was measured at 495 mμ between 7 and 10 min. after the addition of the alkali. Preliminary work with pure γ-coniceine showed that the maximum colour intensity occurred between 7 and 10 min. and the peak of the absorption curve was at 495 mμ. The amount of γ-coniceine present was calculated from a calibration curve prepared from pure γ-coniceine as follows.

Preparation of pure γ-coniceine standard solution. The leaves of second-year *Conium* plants, collected in the spring, were found to be good sources of γ-coniceine. Steam-distillation in the presence of alkali was found unsuitable as the oily distillate was black. The dried leaf was therefore extracted in the cold with chloroform–aqueous NH_3 solution (sp.gr. 0·880) (25:1, v/v). The chloroform solution was washed with water, and was then extracted with 5% (w/v) tartaric acid solution (we found that γ-coniceine turned pink in mineral acid). The pale-yellow acid solution was made alkaline to litmus with aqueous KOH and re-extracted with chloroform; this chloroform solution was then extracted with N-HCl. At this stage we found it impossible to prepare pure crystalline γ-coniceine hydrochloride owing to its deliquescent nature and to the discoloration already referred to. We found, however, that the reineckate was the most suitable salt for further purification. Accordingly a saturated aqueous solution of ammonium reineckate was immediately added to the solution of γ-coniceine in HCl until no further precipitation occurred. The pink flocculent precipitate was dried in a desiccator and pure γ-coniceine reineckate (m.p. 143° uncorrected) obtained by several recrystallizations from acetone–water (25:75, v/v). A portion (20 mg.) of this reineckate was dissolved in 10 ml. of methanol by warming and the solution diluted with an equal volume of water. The reineckate ion was precipitated by titration with aqueous 1% $AgNO_3$ solution until no further precipitate formed. The mixture was filtered, the precipitate well washed with water and the filtrate and washings were made up to 100 ml. This solution contained γ-coniceine (70 μg./ml., calculated as the hydrochloride).

Determination of individual alkaloids (other than γ-coniceine). The method of band chromatography devised by Cromwell was applied to the solution of alkaloidal hydrochlorides already referred to. As this method is laborious it was used only when information on the quantities of alkaloids other than γ-coniceine and coniine was required.

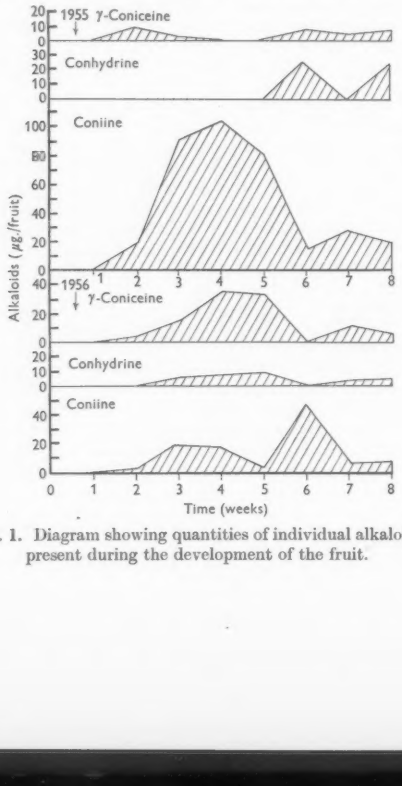


Fig. 1. Diagram showing quantities of individual alkaloids present during the development of the fruit.

RESULTS

Assay of alkaloids

The results are expressed as weight of alkaloid per fruit, rather than percentage of dry or fresh weight, as this gave a more accurate picture of the changes taking place within an individual fruit. The results for 1955 and 1956 are summarized in Fig. 1.

Effect of rainfall and sunshine. The 1955 season was sunny and dry with a total sunshine of 349 hr. and rainfall of 1·16 in. during the 8 weeks of the experiment. In 1956 the weather was less sunny and wetter with a total sunshine of 221 hr. and rainfall of 8·66 in. during the corresponding 8 weeks. In 1955 the average dry weight of the fruits and the amount of alkaloid per fruit was about twice that of the 1956 season. Other effects on the composition of the alkaloids are discussed below.

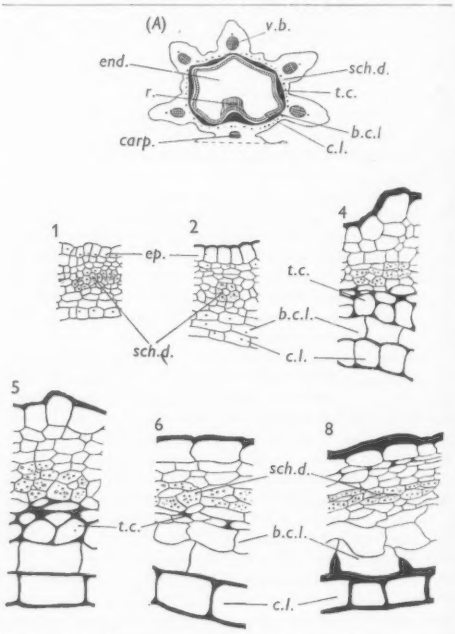


Fig. 2. (*A*). Tissue distribution, as seen in transverse section, of a mericarp at week 5 (×15). 1, 2, 4, 5, 6, 8, (numbers indicate the week of collection): tissue details of the pericarp (×200). *b.c.l.*, Beaker-cell layer; *carp.*, carpophore; *c.l.*, coniine layer; *end.*, endodermis; *ep.*, epidermis; *r.*, raphe; *sch.d.*, schizogenous duct; *t.c.*, temporary collenchyma; *v.b.*, vascular bundle.

Table 1. *Summary of anatomical changes during development of the fruits in* 1955

Week no.	Coniine layer	Beaker-cell layer	Temporary collenchyma	Schizogenous ducts	Endosperm
1, 2	Walls not thickened	Walls not thickened	Absent	Present	Translucent
3	Inner tangential walls partially thickened	Walls not thickened	Absent	Present	Translucent
4	Both tangential walls partially thickened	Inner tangential walls partially thickened	Developing	Present	Translucent
5	Radial and tangential walls thickened	Inner tangential walls partially thickened	Fully developed	Fully developed	Becoming opaque; minute crystals appear
6	Radial and tangential walls thickened	Inner tangential walls partially thickened	Partially lost. Some distortion	Tangential compression begins	Opaque
7	Walls completely thickened	'Beaker-shaped' thickening	Lost and tissues distorted	Distorted	Opaque; crystals at maximum size
8	Walls completely thickened	'Beaker-shaped' thickening	Lost and tissues distorted	Distorted	Opaque

Developmental anatomy of the fruit

The general arrangement of the tissues, as seen in transverse section, is shown in Fig. 2. The endocarp (coniine layer) and the layer of pericarp cells immediately within (which we have called the 'beaker-cell layer') have long been known to contain alkaloids (Goris, 1914). Both develop at approximately the same rate but the ultimate thickening and pitting of the walls indicate that diffusion of substances from these layers would be outwards towards the mesocarp rather than inwards towards the seed (Fig. 2, 8). In the inner region of the mesocarp, temporary collenchyma arises in week 4 and disappears in week 7. During this same period the endosperm tissues reach maturity both in size and cell contents. There are numerous schizogenous ducts (vittae) in the mesocarp but at about week 4 they cease to develop and never form well-marked vittae which are so characteristic of most umbelliferous fruits.

A summary of these changes is given in Table 1 and some of the stages are illustrated in Fig. 2.

Histochemical studies

We used a solution of potassium bismuth iodide (Munier, 1951) as a general histochemical reagent for alkaloids, and an alkaline solution of sodium nitroprusside for piperideines. The latter was used by placing a thick section (about 0·5 mm.) in a drop of freshly prepared 1 % sodium nitroprusside solution on a slide, followed by the addition of a drop of aqueous 10 % NH_3 solution; if piperideines, such as γ-coniceine, were present a blood red developed. Fully saturated alkaloids like coniine and conhydrine do not give red.

Alkaloid was found in the epidermis and in the coniine layer at all stages of development, being particularly abundant about week 5, but its presence was doubtful in all other tissues. This observation conflicts with that of earlier workers, whose results are reviewed by Goris (1914). He states that coniine occurs in the epidermis, subepidermal collenchyma, parenchyma adjoining the vascular bundles and schizogenous ducts and in the coniine layer and its adjacent one (the beaker-cell layer). It is completely absent from the seed. Our own results do not prove that coniine is totally absent from the other tissues, as Tunman (1931) reports that potassium bismuth iodide is not sensitive to *Conium* alkaloids in concentrations of less than 0·1 %. They do indicate, however, that the alkaloids are concentrated in the peripheral tissues of the pericarp, that is, the epidermis and the endocarp.

The distribution of piperideines showed considerable variation. Well-marked reactions were obtained in the vascular bundles of the carpophore and pericarp during the entire development of the fruit. In the raphe and the schizogenous ducts piperideines were present in the early stages, being particularly intense in the ducts in week 4, but only traces were present in the later stages. None occurred in the coniine and beaker-cell layers until week 6 and even then the reaction in the coniine layer was doubtful. This appearance of piperideines in the beaker-cell layer coincided with a general diffusion of piperideines throughout the pericarp tissues from week 6 onwards (Table 2).

Aborted mericarps. Abnormal fruits occurred fairly frequently; in these, one mericarp developed normally and the other became aborted. Histochemical tests showed that piperideines were always much more abundant in the aborted mericarp than in the normal one.

Table 2. *Distribution of piperideines in fruits collected in* 1956, *as shown by the nitroprusside test*

Colours: ±, doubtful red; +, blood red. The tissues referred to are shown in Fig. 2.

Week no.	1	2	3	4	5	6	7	8
Epidermis	.	.	.	.	.	.	+	+
Schizogenous ducts	+	+	+	+	+	+	±	±
Collenchyma	.	.	.	.	.	+	+	+
Beaker-cell layer	.	.	.	.	.	+	+	+
Coniine layer	.	.	.	.	.	+	±	±
Vascular bundles in pericarp and carpophore	+	+	+	+	+	+	+	+
Raphe and adjoining parenchyma	.	+	+	+	+	+	±	±

DISCUSSION

Piperideines as precursors of the saturated alkaloids

The results illustrated in Fig. 1 show that in both seasons a peak of γ-coniceine content nearly always preceded a peak of coniine content. This happened twice in 1955 and shows particularly well in 1956, between weeks 5 and 6, when the γ-coniceine dropped rapidly and the coniine increased correspondingly. These results are in agreement with those of Cromwell (1956), who found that in growing seedlings and young leaves γ-coniceine predominated but that as the vegetative parts matured so the content of coniine increased. Similarly, in the development from flower to fruit the overall picture was of the γ-coniceine content decreasing as the coniine content increased. Therefore γ-coniceine may well be the precursor of coniine. The results of our histochemical studies are also in agreement with this hypothesis, since they indicate that piperideines enter the fruit by the conducting tissues and the schizogenous ducts and that in the latter they appear to reach a maximum concentration in week 4, the same week as that in which total alkaloids are also at a maximum.

Interchange between saturated and unsaturated alkaloids

γ-Coniceine seems to be the predominant alkaloid during rainy weather. Thus in both seasons the maximum alkaloidal content was at week 4, but in the comparatively dry conditions of 1955 coniine was the predominant alkaloid whereas in the wetter season of 1956 γ-coniceine predominated at the corresponding stage. Further, between weeks 5 and 6 in 1956 there was a marked reduction in the γ-coniceine content (and a corresponding increase in coniine). This was paralleled by a very marked reduction of rainfall. Since the overall effect of the dry 1955 season was an increase in growth it may well be that the change from γ-coniceine to the saturated alkaloids is associated with active growth. This is supported by a consideration of the anatomical studies, which also indicate that this change is reversible.

Anatomical studies of the 1955 samples showed that the rapid increase in alkaloidal content occurred while the pericarp was increasing in size, up to weeks 4 and 5. During this time piperideines were abundant in the schizogenous ducts (which are possibly important sources of alkaloid precursors) and vascular tissue; the coniine and beaker-cell layers increased in size but gave reactions for saturated alkaloids only. Presumably piperideines are transported to the pericarp during this period of rapid development, are hydrogenated or hydrated and then deposited in the coniine and beaker-cell layers as saturated alkaloids. In the aborted mericarp, on the other hand, no such rapid development takes place and the piperideines accumulate to a much greater extent than in the corresponding normal mericarp. After week 4 the alkaloidal content falls. This is paralleled by the gradual distortion of the schizogenous ducts and a reduction in their piperideine content. At the same time the seed begins to mature and it may be in connexion with this further metabolic activity that additional changes take place in the alkaloidal content. Piperideines begin to appear in the coniine and beaker-cell layers and become generally diffused throughout the pericarp (Table 2). Chemical analyses confirm that there is an increase in γ-coniceine from weeks 5 to 6 (Fig. 1). These results indicate that the 'piperideine pool' associated with this second phase of metabolic activity is partly derived from the stores of coniine in the coniine and beaker-cell layers. It has already been pointed out that the thickening of the walls of these cells would favour a diffusion from them into the pericarp. The conversion of piperideines into saturated alkaloids may therefore be reversible. A similar situation has been shown to occur in certain *Senecio* species, where the alkaloids occur either in an oxidized or a reduced form according to reversible reactions of hydrogenation and varying degrees of hydration (Areschkina, 1957). We intend investigating further this possibility that the hemlock alkaloids are connected with oxidation–reduction mechanisms.

Assay figures for total alkaloids

We found that in both seasons the maximum percentage of alkaloids (dry-weight basis) was about 3%. This is considerably higher than the figures usually quoted (Farr & Wright, 1904; Madaus & Schindler, 1938; Cromwell, 1956), and is probably due to the careful sampling technique we employed. A sample collected haphazardly from several plants on any given day will contain fruits at many stages of development and this would lead to a lowering of the assay value. Farr & Wright (1904) state that the highest percentage of alkaloids occurs when the fruit is green, and the results given by Cromwell (1956) indicate a maximum in mature green fruits. Our results show that these statements should be modified, for the maximum occurred in 1955 when the fruits were just beginning to turn yellow, whereas in 1956 the fruits were still quite green after the period of alkaloidal maximum. In 1955 the total alkaloid per fruit was twice that of the fruit in 1956; at the same time the dry weight was also doubled so that the assay figures for the two seasons were similar. Increased alkaloidal production is therefore associated with the general improvement in growth and vigour.

SUMMARY

1. A study of the changes occurring during the development of the flower to the fruit of *Conium maculatum* L. has been made.

2. The changes in the composition and quantity of alkaloids were investigated simultaneously with the changes in anatomical structure and in reactions to histochemical tests.

3. A carefully chosen sample of seeds was used and crops from two successive seasons were harvested. Special sampling techniques and methods of analysis were devised.

4. The climatic conditions in the two seasons differed; in the sunnier season the average weight of the fruits and amount of alkaloids was twice that of the wetter season.

5. In both seasons the maximum alkaloidal content occurred 3 weeks after fertilization of the ovule; in the wet season, however, γ-coniceine (an unsaturated alkaloid) predominated at this stage; in the dry season coniine (a saturated alkaloid) predominated.

6. In both seasons a peak of γ-coniceine content preceded a peak of coniine content.

7. It is suggested that γ-coniceine and other piperideines are the precursors of the saturated alkaloids such as coniine and conhydrine and this change is associated with active growth. This change may be reversible.

The work described in this paper forms part of a thesis presented by one of us (S. B. Challen) for the award of a Ph.D. degree of the University of London. We would like to thank Mr J. F. Rogers, Head Gardener at Myddelton House, for his valuable assistance in the cultivation of the hemlock plants.

REFERENCES

Areschkina, L. Y. (1957). *Biokhimiya*, **22**, 527.
Barreto, A. (1954). *Agronomia, Rio de J.*, **13**, 45.
Cromwell, B. T. (1956). *Biochem. J.* **64**, 259.
Dawson, R. F. (1948). *Advanc. Enzymol.* **8**, 203.
Dilling, W. J. (1909). *Pharm. J.* (IV), **29**, (IV), **34**, 70, 102.
Euler, U. S. von (1945). *Acta pharm. tox., Kbh.*, **1**, 29.
Farr, E. H. & Wright, R. (1904). *Pharm. J.* (IV), **18**, 185.
Fisher, R. B., Parsons, D. S. & Holmes, R. (1949). *Nature, Lond.*, **164**, 183.
Fisher, R. B., Parsons, D. S. & Morrison, G. A. (1948). *Nature, Lond.*, **161**, 764, 765.
Goris, A. (1914). *Localization et Role des Alcaloides et des Glucosides*, p. 116. Paris: Lechevalier.
Janot, H. & Fabre, C. (1929). *Bull. Sci. pharm.* **36**, 529.
Madaus, G. & Schindler, H. (1938). *Arch. Pharm., Berl.*, **276**, 280.
Munier, R. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 846.
Reichard, C. (1905). *Pharm. Zentralh.* **46**, 252.
Tunman, O. (1931). *Pflanzen-Mikrochemie*, p. 315. Berlin: Borntraeger.

The Point of the Aerobic Inhibition of Glycolytic Activity Associated with Brain Mitochondria

By R. BALÁZS
Medical Research Council Neuropsychiatric Research Unit, Whitchurch Hospital, Cardiff

(*Received* 25 *August* 1958)

One of the oldest-known controlling mechanisms of metabolic processes at the cellular level is the aerobic sparing effect on glucose utilization. Warburg (1926) defined this effect as follows: 'Respiration and glycolysis are linked by a chemical reaction which I call the Pasteur reaction after its discoverer.' This reaction has been the subject of a large number of investigations (cf. reviews by Burk, 1937, 1939; Dixon, 1937; Lipmann, 1942; Dickens, 1951; Krebs, 1957). However, the elucidation of the mechanism and of the point of inhibition in the glycolytic chain has been hampered by the fact that the effect was obtained only in whole-cell preparations, where permeability factors

limited the scope of the investigations. Recent progress in the study of multi-enzyme systems now permits a more detailed investigation of the enzymic mechanisms operating in the rate-control of metabolic processes.

In recent years there have appeared a few investigations of the Pasteur effect in cell-free preparations (Birmingham & Elliott, 1951; Ács *et al.* 1955; Terner, 1954, 1956; Gatt, Krimsky & Racker, 1956; Aisenberg, Reinafarje & van Potter, 1957; Aisenberg & van Potter, 1957; Gatt, 1957; Wu & Racker, 1957; Tiedemann & Born, 1958).

In the present study this effect was investigated in brain-mitochondria preparations with which part of the cell glycolytic activity was found to be associated, as previously reported by Hesselbach & Du Buy, 1953; Gallagher, Judah & Rees, 1956; Strecker, 1957. It is not known whether the glycolytic system is inherently part of the brain mitochondria or whether it is simply adsorbed on the particles contained in the mitochondrial fraction. However, the finding that about 10% of the glycolytic activity in brain homogenates is consistently associated with the mitochondrial fraction (Balázs & Lagnado, 1959) makes this preparation a convenient experimental material for studying the metabolic processes in which respiration and glycolysis are associated.

The results of this investigation demonstrate that glycolytic activity associated with brain mitochondria is inhibited under aerobic conditions and that the principal site of this inhibition is at the triose phosphate dehydrogenase step. Some of these results have been reported in a preliminary communication (Balázs & Richter, 1958).

EXPERIMENTAL

Preparation of brain mitochondria. Wistar albino rats weighing 120–230 g. were used. Brain mitochondria were separated from 10% homogenates prepared with a NIRD homogenizer (Nelco Ltd., Shalford, Surrey) by a method similar to that originally used by Brody & Bain (1952) (fraction $1R_3$). The medium for homogenization was 0·25 M-sucrose solution containing 25 mM-nicotinamide. As reported elsewhere (Balázs & Lagnado, 1959), the glycolytic activity (as well as that of several glycolytic enzymes) associated with the mitochondrial fraction after one washing with the sucrose medium reached a final level which was not changed by two subsequent washings. In most of the present experiments, the 'fluffy layer' which remains above the mitochondrial sediment was retained; the results were not modified whether it was kept or removed (Balázs & Lagnado, 1959). It was found that this preparation gave reproducible results in studies of the changes in glycolytic activity under different experimental conditions.

The mitochondrial sediment was suspended in, and diluted with, a salt solution of the following composition: 100 ml. of 0·154 M-NaCl, 1·0 ml. of 0·154 M-$MgSO_4$, 5·25 ml. of 0·5 M-nicotinamide, 10·0 ml. of 0·1 M-potassium phosphate buffer, pH 7·4. Replacement of NaCl by KCl did not alter the results. The entire procedure was carried out at 0–5° and the mitochondria were used within 30 min. of preparation.

Reaction mixture. The standard medium contained 6 mM-$MgSO_4$, 0·01 M-potassium phosphate buffer, pH 7·4 (0·1 M-KH_2PO_4 solution was adjusted with KOH to pH 7·4 as determined with Cambridge 44236 bench-pattern pH meter), 0·01 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris), pH 7·4, 2 mM-ethylenediaminetetra-acetate (EDTA) and 25 mM-nicotinamide after the addition of all adjuvants. Further additions to the standard medium are specified below. The pH of tricarboxylic acid-cycle intermediates and the adenine nucleotides was adjusted to pH 7·0–7·4 with KOH before addition. The final concentration of K^+ ion was 0·01–0·05 M (LePage, 1948). The final volume in the flasks was 1·5–3·0 ml. The osmolarity of the medium was adjusted with 0·154 M-KCl to approx. 0·3–0·32 (Aldridge, 1957).

Incubation. Incubations were carried out in conventional Warburg vessels at 37·5°. For the anaerobic experiments the vessels were gassed with N_2 (freed from traces of O_2 by passage through a heated copper tube), and the 'aerobic' experiments were in air. The incubation time was usually 30–40 min. Both respiration and glycolysis usually remained linear during this period. When the consumption of O_2 was measured (by the Warburg technique; Umbreit, Burris & Stauffer, 1945), the vessels contained 0·1 ml. of 20% (w/v) KOH in the centre well, temperature equilibration was carried out with open stopcocks for 6–10 min. and results were extrapolated for this pre-incubation period unless otherwise stated.

The reaction was stopped in most cases with perchloric acid (final concentration 0·3 M). Trichloroacetic acid (final concentration 5%) was used when keto acids were to be determined. When glucose and lactate were to be determined simultaneously, samples from the incubation mixture were precipitated directly with the Somogyi reagent (Somogyi, 1945) and with the $CuSO_4$–$Ca(OH)_2$ mixture (Barker & Summerson, 1941) respectively.

Analytical methods. Glucose and lactate were determined by the methods of Nelson (1944) and Barker & Summerson (1941) respectively. In the lactate determination the samples were precipitated in the $CuSO_4$–$Ca(OH)_2$ mixture at 0° and kept in an ice bath for 30 min. This prevented the breakdown of triose phosphates to products which would be determined as lactate.

Pyruvate and total keto acids were measured by the method of Friedemann & Haugen (1943). The spectra of the pyruvate- and α-oxoglutarate-2:4-dinitrophenylhydrazones were measured between 420 and 650 mμ, and in the photometer used (Unicam SP. 350, model D.G. spectrophotometer) these gave the maxima at 480 and at 450 mμ respectively. Regular measurements of extinction were made at 480 and 440 mμ. The pyruvate-2:4-dinitrophenylhydrazones were extracted for 5 min. with benzene and the total keto acids for 25 min. with ethyl acetate.

Inorganic phosphate was determined in perchlorate or trichloroacetic acid filtrate by the Fiske–Subbarow method as modified by Lohmann & Jendrassik (1927). When the amount of phosphate to be measured was below 10 μg. the method of Berenblum & Chain (1938) was used.

Triose phosphates were estimated by the method of Meyerhof & Lohmann (1934) (hydrolysis for 20 min. in

N-NaOH at 25°). Hexose diphosphate (FDP) was measured by the method of Kiessling (1938) (hydrolysis for 3 min. in 0·2N-NaOH at 100°). (In the absence of hexose monophosphates, as in these experiments, the method is valid for the determination of FDP.) In these two methods, based on alkali-lability, the adenine nucleotides were not hydrolysed under the conditions used; thus the determination of FDP and triose phosphates could be carried out without prior removal of nucleotides.

Chromatography and radicautography. The glycolysis intermediates were separated by paper chromatography. The procedure used was as follows: nucleotides were removed, by the method of Crane & Lipmann (1953), by treating the perchloric acid filtrate with Norite SX-Special. The resulting filtrates were made up to a known volume, adjusted with 0·3N-$Ba(OH)_2$ to pH 8·4–9·2 (pink to phenolphthalein) and precipitated with 4·5 vol. of cold 95% ethanol. They were left at −10° overnight. The precipitate obtained after centrifuging was suspended in water and the barium was removed by shaking with Amberlite IR 120 H^+ ion-exchange resin, yielding an almost clear solution. This solution, together with aqueous washings of the resin, was then neutralized with ammonia and evaporated to dryness at 35–40° by vacuum-distillation. The dried material was dissolved in a known volume of water, made slightly alkaline (pH 9·0) with ammonia and spotted on the paper for chromatography.

Chromatography was carried out according to the method of Hanes & Isherwood (1949) on acid-washed Whatman no. 1 filter paper. The most suitable solvent systems found were ethyl acetate–acetic acid–water (4:3:1, by vol.; Mortimer, 1952) for the first direction and propanol–aq. NH_3 soln.–water (6:1:3, by vol.; Hanes & Isherwood, 1949) for the second. The use of these solvent systems make it unnecessary to remove the inorganic phosphate to obtain a good separation of the compounds. Control markers were always run. The chromatograms were sprayed and the spots were developed according to the method of Hanes & Isherwood (1949), except that u.v. light was used to produce the colour of the spots (Bandurski & Axelrod, 1951). The recovery of pure FDP, glucose 6-phosphate (G 6-P) and 3-phosphoglycerate was 80–90% with this method.

The amount of ^{32}P (The Radiochemical Centre, Amersham, Bucks) added per vessel was 7–15 μc. Chromatograms were made as described above and the dried papers were used for radioautography. The starting lines and the direction of the solvents were marked with radioactive ink (Benson *et al.* 1950) and the paper was placed on Blue-Brand X-ray film in a Kodak X-ray Translucent Cassette with a Kodak type 80 Intensifying Screen for 24 hr. After development of the X-ray films, the corresponding spots were cut out and eluted (Dent, 1947). The eluate was made to a known volume and separate portions were used for identifications and for estimations of P. Only FDP and G 6-P were identified. In other experiments the active spots as well as paper blanks were cut out and digested with perchloric acid.

Phosphoglycerate determination. In the measurement of phosphoglycerate (PGA) it was necessary to eliminate the interference caused by the high content of inorganic phosphate, FDP, triose phosphates and nucleotide P. The determination was based on the assumption that under the experimental conditions used (FDP as substrate instead of glucose) PGA was the only compound formed which contained stable phosphate. This assumption was verified by chromatography. The determination was carried out as follows. Nucleotides were removed from the perchloric acid filtrate by Norite. The FDP and triose phosphates were hydrolysed in N-NaOH for 10 min. in a boiling-water bath (leaving the PGA unhydrolysed). Inorganic phosphate was precipitated with a magnesium mixture (Umbreit *et al.* 1945) and the stable phosphates were determined in the supernatant. In a few experiments the inorganic phosphate was precipitated according to the method of Lipmann & Tuttle, 1944.

It was found by chromatography that the stable phosphate remaining after this treatment was PGA. After the perchloric acid filtrate was treated as mentioned above, the inorganic phosphate-free supernatant was precipitated with barium and ethanol after adjusting the pH to 8·4–9·0, and the precipitate was prepared for chromatography (cf. above). Chromatograms were run in ethyl acetate–acetic acid–water and also as described by Cowgill (1955). For the latter method Whatman no. 1 unwashed paper gave better results in our hands than the Whatman no. 52 recommended by the author. The chromatograms have revealed three spots corresponding (*a*) to inorganic phosphate (small spot), (*b*) to 3-phosphoglycerate and (*c*) to 2-phosphoglycerate. This last spot was spread over a large area.

RESULTS

Accumulation of lactate in the mitochondrial fraction of brain homogenates under aerobic and anaerobic conditions

About 10% of the glycolytic activity of brain homogenates was found to be associated with the mitochondrial fraction, as indicated by the formation of lactate (in a glucose-containing medium) (Balázs & Lagnado, 1959). An investigation of the influence of aerobic conditions on the glycolytic activity associated with the mitochondrial fraction revealed that lactate production was inhibited aerobically, the extent of the inhibition being dependent on the experimental conditions used. This is illustrated in Tables 1 and 2. When only glucose was added to the standard medium a large aerobic inhibition of lactate accumulation was observed ($83 \pm 2{\cdot}5$ %). This could be due (*a*) to the oxidative removal of pyruvate formed during glycolysis or (*b*) to an inhibition in the glycolysis cycle. The addition of pyruvate, which could spare the end product of glycolysis, did not alleviate the aerobic inhibition of lactate accumulation (Tables 2 and 3). Furthermore, it was found that the glucose consumption was also decreased under aerobic conditions (Table 3, *a* and *c*). If glucose disappearance proceeded at the same rate, and the only difference between the aerobic and anaerobic systems was in the relative amounts of pyruvate reduction, no decrease in glucose consumption would be expected. These findings demonstrate that the main effect of aerobic conditions is an inhibition in the glycolysis reactions.

Effects of tricarboxylic acid-cycle intermediates

In the following experiments the influence of oxidative substrates and of cofactors on glucose metabolism in mitochondrial preparations was investigated. The effects of all the Krebs-cycle intermediates tested were similar (Tables 1, 3; Table 4, Expt. 5; Table 10, Expt. 1).

The addition of Krebs-cycle intermediates did not change the percentage inhibition of aerobic lactate formation ($80 \pm 3{\cdot}1$ instead of $83 \pm 2{\cdot}5$%; Table 2). However, in the presence of adenosine

Table 1. *Effect of cofactors and tricarboxylic acid-cycle intermediates on the aerobic inhibition of lactate formation in brain-mitochondria preparations*

System: standard medium; 0·01 M-glucose. Additions: 2 mM-ATP, 0·33 mM-DPN; 3 mM-pyruvate and 2 mM-L-malate (Expt. 1), 6·7 mM-α-oxoglutarate (Expt. 2). Brain mitochondria were added, equivalent to 140 mg. of fresh tissue. Volume, 1·5 ml. Duration of experiment, 35 min. (Expt. 1), 30 min. (Expt. 2). Percentage inhibition of aerobic lactate production: $\frac{\text{lactate}_{N_2} - \text{lactate}_{air}}{\text{lactate}_{N_2}} \times 100$.

Expt. no.	Additions	Lactate formed (μmoles) Aerobic	Anaerobic	Inhibition (%)
1	Pyruvate, L-malate, DPN	0·26	1·35	81
	ATP, DPN	0·50	1·62	69
	Pyruvate, L-malate, ATP, DPN	1·63	2·25	28
2	DPN	0·14	0·50	72
	DPN, α-oxoglutarate	0·17	0·60	72
	ATP, DPN	0·47	0·77	39
	ATP, DPN, α-oxoglutarate	0·69	0·91	24

Table 2. *Effect of tricarboxylic acid-cycle intermediates and cofactors on the inhibition of aerobic lactate production in brain mitochondria*

System: standard medium; glucose (5–10 mM). Additions: (*a*) Krebs-cycle intermediates [3 mM-pyruvate and 2 mM-L-malate (35 expts.), or 5–10 mM-α-oxoglutarate (15 expts.) or 10 mM-L-glutamate (1 expt.)]; (*b*) cofactors [2 mM-adenine nucleotide (ATP, ADP or AMP), 0·33 mM-DPN]. The reaction was started by addition of mitochondrial suspension. Incubation was for 30–50 min. The amount of mitochondria added was different from experiment to experiment; in general it was equivalent to 150–1000 mg. of fresh brain. Lactate figures represent means.

Additions	No. of expts.	Lactate formed (μmoles/g. of fresh brain equivalent/30 min.) Air	N_2	Aerobic inhibition (%)
Glucose	8	0·39	2·45	$83 \pm 2{\cdot}5$
Glucose, Krebs-cycle intermediates	13	0·85	4·50	$80 \pm 3{\cdot}1$
Glucose, cofactors	16	2·54	6·05	59 ± 4
Glucose, Krebs-cycle intermediates, cofactors	37	4·67	7·50	$39 \pm 2{\cdot}3$

Table 3. *Effect of cofactors on glucose metabolism in brain mitochondria*

System: standard medium; additions as indicated in the table. Brain mitochondria were added equivalent to 280 mg. of fresh tissue. Volume, 1·42 ml. Duration of experiment, 40 min.

	O_2 consumed (μmoles)	Glucose consumed (μmoles) Air	N_2	Lactate formed (μmoles) Air	N_2	Inhibition (%)	Pyruvate consumed (μmoles) Air	N_2	Other keto acids formed (μmoles) Air	N_2
Gas phase										
Additions (μmoles)										
(*a*) Glucose (5·1), pyruvate (9), L-malate (5·7)	3·37	0·25	1·42	0·59	3·14	81	2·27	0	0	0
(*b*) Glucose (5·1), pyruvate (9), L-malate (5·7), ATP (2·84), DPN (0·47)	15·6	4·95	2·35	3·18	5·45	41	8·33	0	4·23	0
(*c*) Glucose (5·1), α-oxoglutarate (11·4)	2·66	0·38	1·41	0·43	2·88	85	0	0	−1·7	(−0·2)
(*d*) Glucose (5·1), α-oxoglutarate (11·4), ATP (2·84), DPN (0·47)	—	4·65	2·15	2·72	4·54	40	0	0	−9·2	0

triphosphate (ATP; 2 mM) and diphosphopyridine nucleotide (DPN; 0·33 mM), which partly relieved the inhibition (59 ± 4 %), the addition of oxidative substrates further alleviated the aerobic inhibition of lactate formation (39 ± 2·3 %; Table 2). The quantitative results of two representative experiments are illustrated in Table 1. In a fortified system, the addition of oxidative intermediates increased both the aerobic and anaerobic lactate production. However, since in the aerobic system the increase was relatively larger, the inhibition was decreased.

It was found that the extra lactate production in the presence of added pyruvate or α-oxoglutarate could not be accounted for by a dismutation reaction (Krebs & Johnson, 1937; Weil-Malherbe, 1937), because little or no lactate was formed in the absence of added glucose (probably derived from endogenous glycolysis substrates). Moreover, no keto acid consumption was detected under anaerobic conditions in the presence of glucose (Table 3).

Thus, as was previously suggested by Mendel, Bauch & Strelitz (1931) and by Elliott & Henry (1946), the increased lactate production could be due to a stimulation of glycolysis by pyruvate or traces of pyruvate metabolically derived from other Krebs-cycle intermediates.

Effects of cofactors

The effects of ATP and DPN on glucose metabolism in brain mitochondria are shown in Table 4. In Expts. 1–3, glucose was the only added substrate; in Expts. 4 and 5 Krebs-cycle intermediates were also included. The effect of cofactors (ATP, DPN) was essentially the same in the two systems. The low respiration found without added cofactors was markedly stimulated by the joint addition of ATP and DPN. The lactate production and glucose consumption in the unfortified systems were inhibited under aerobic conditions (cf. also glucose-consumption values in the presence of Krebs-cycle intermediates, Table 3). With the separate addition of ATP and DPN the aerobic inhibition of lactate production remained high though glucose consumption was generally greater aerobically than anaerobically. The joint addition of both cofactors markedly decreased the aerobic inhibition of lactate production, and the aerobic glucose consumption greatly exceeded that observed anaerobically.

The effects of varying concentrations of ATP and adenosine diphosphate (ADP) in a system fortified with α-oxoglutarate and DPN were essentially the same (Fig. 1). When a concentration of 2 mM was reached, glycolysis was increased and the aerobic

Table 4. *Effect of cofactors on glucose metabolism in brain mitochondria*

System: standard medium; substrates as indicated in the table. Cofactors: 2 mM-ATP, 0·33 M-DPN. Final volume, 1·5 ml. Mitochondria added (equivalent to mg. of fresh tissue) and incubation time: Expt. 1, 200, 35 min.; Expt. 2, 300, 40 min.; Expt. 3, 200, 40 min.; Expt. 4, 200, 35 min.; 160, 35 min.

Expt. no.	Substrates (μmoles)	Additions	O_2 consumed (μmoles)	Glucose consumed (μmoles)		Lactate formed (μmoles)		Inhibition (%)
	Gas phase			Air	N_2	Air	N_2	
1	Glucose (15)	—	—	—	—	0·26	0·92	71
		DPN	0·86	—	—	0·25	1·17	79
		ATP	1·13	—	—	0·33	1·19	73
		ATP, DPN	—	—	—	0·55	1·62	66
2	Glucose (4·54)	—	0·78	0·21	0·38	0·06	0·57	89
		DPN	0·76	0·63	—	0·03	0·82	96
		ATP	—	0·88	0·40	0·15	0·75	81
		ATP, DPN	1·21	1·13	0·55	0·62	1·09	43
3	Glucose (4·7)	—	0·51	0·25	0·45	0·09	0·52	83
		DPN	0·80	0·72	—	0·14	0·72	81
		ATP	1·34	0·96	0·43	0·11	0·72	85
		ATP, DPN	3·57	1·55	0·55	0·47	0·96	51
4	Glucose (15), pyruvate (9), L-malate (3)	—	0·41	—	—	0·40	2·58	85
		DPN	1·89	—	—	0·46	2·66	83
		ATP	2·21	—	—	0·69	3·25	72
		ATP, DPN	9·2	—	—	2·66	3·38	22
5	(*a*) Glucose (15), pyruvate (4·5), L-malate (3)	—	0·54	—	—	0·17	0·85	80
		DPN	0·58	—	—	0·26	0·90	72
		ATP	—	—	—	0·48	1·09	57
		ATP, DPN	3·59	—	—	1·10	1·55	29
	(*b*) Glucose (15), L-glutamate (15)	ATP, DPN	—	—	—	0·77	1·02	25

inhibition of lactate formation decreased, from 92% to 28 and 21% with ATP and ADP respectively. The effect of adenosine monophosphate (AMP) was qualitatively similar to that of ATP and ADP.

The effects of varying concentrations of DPN were tested in the presence of Krebs-cycle intermediates and 2 mM-ATP. The optimum concentration of DPN was found to be 0·33 mM. Triphosphopyridine nucleotide (TPN) in the same concentration did not appreciably promote the metabolism of glucose.

Fortifying the system with optimum concentrations of adenine nucleotides and DPN resulted in a high aerobic glucose consumption (exceeding two to six times the anaerobic) and in a partial decrease of the aerobic inhibition of lactate production (inhibition remaining $39 \pm 2{\cdot}3$%). The theoretical amount of glucose consumed, which could be calculated from the extra oxygen consumption in the presence of glucose, together with the amounts of lactate which accumulated, could not account for the large increase observed in the disappearance of glucose. This indicates an inhibition occurring in the glycolysis reactions, which could result in the accumulation of intermediates of glycolysis.

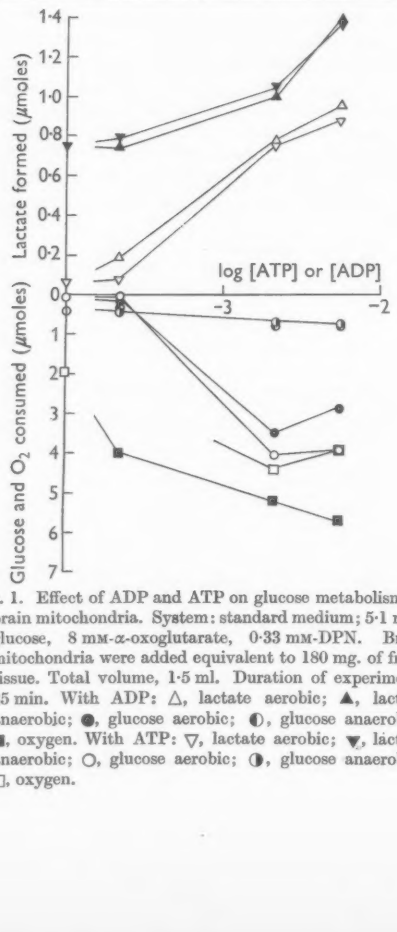


Fig. 1. Effect of ADP and ATP on glucose metabolism of brain mitochondria. System: standard medium; 5·1 mM-glucose, 8 mM-α-oxoglutarate, 0·33 mM-DPN. Brain mitochondria were added equivalent to 180 mg. of fresh tissue. Total volume, 1·5 ml. Duration of experiment, 35 min. With ADP: △, lactate aerobic; ▲, lactate anaerobic; ●, glucose aerobic; ◐, glucose anaerobic; ■, oxygen. With ATP: ▽, lactate aerobic; ▼, lactate anaerobic; ○, glucose aerobic; ◑, glucose anaerobic; □, oxygen.

Inhibition of aerobic lactate formation with different intermediates of glycolysis

To test the possibility of a block in the glycolysis chain, experiments were performed in which glucose was replaced by the glycolysis intermediates FDP and G 6-P. In the experiment shown in Table 5 the glycolysis substrates were tested in the presence of cofactors. The highest glycolytic activity was obtained with FDP. The aerobic inhibition of lactate production was 66% with glucose, 32% with G 6-P and 19% with FDP. The results suggested that a part of the inhibition took place at the level of the phosphorylation of glucose and that a part of the inhibition occurred at a lower level. In the experiments shown in Table 6 the effect of Krebs-cycle intermediates and cofactors on the aerobic inhibition of glycolysis were investigated in the presence of different glycolysis intermediates. G 6-P acted very similarly to glucose, except that the aerobic inhibition observed under each of the conditions tested was less. This was not the case with FDP. When glucose was used as the substrate, the addition of cofactors and Krebs-cycle intermediates decreased the aerobic inhibition of lactate accumulation. On the other hand, with FDP as substrate the addition of the same factors caused an increase in the aerobic inhibition. After pooling data from several experiments, it was found that the percentage inhibition of aerobic lactate formation in the presence of cofactors and Krebs-cycle intermediates was $39 \pm 2{\cdot}35$ (37 experiments) with glucose, and $35 \pm 1{\cdot}74$ (11 experiments) with FDP as substrate.

Site of the aerobic inhibition of lactate formation

Since the experimental results suggested an inhibition in the glycolysis reactions, attempts were made to detect an accumulation of intermediates.

Table 5. *Inhibition of aerobic lactate formation with different intermediates of glycolysis*

System: standard medium; glycolytic substrate as indicated in the table. Cofactors: 2 mM-ATP, 0·33 mM-DPN. Mitochondria added were equivalent to 170 mg. of fresh brain. Volume, 1·5 ml. Duration of experiment, 30 min.

	Lactate formed (μmoles)		Inhibition
Gas phase	Air	N_2	(%)
Glycolysis substrate (μmoles)			
Glucose (15)	0·5	1·48	66
Glucose 6-phosphate (15)	0·81	1·18	32
Hexose diphosphate (15)	2·50	3·10	19

Table 6. *Effect of cofactors and tricarboxylic acid-cycle intermediates on the aerobic inhibition of lactate formation with different intermediates of glycolysis*

System: standard medium (inorganic phosphate concentration in Expt. 4, 6 mM); glycolysis substrate in part *A* 10 mM-glucose, in Expt. 1 of part *B* 10 mM-G 6-P, in Expts. 2, 3 and 4 FDP [concn. 10 mM except in Expt. 4 (5·1 mM)]. Krebs-cycle intermediates: 3 mM-pyruvate, 2 mM-L-malate, except in Expt. 4 where α-oxoglutarate was added (8·6 mM). Cofactors: 0·33 M-DPN, 2 mM-ATP, except in Expt. 4 where the same concentration of AMP was added. Mitochondria were added equivalent to 160 mg. of fresh tissue. Incubation time, 30 min., except in Expt. 1 where it was 35 min. Figures represent lactate formation (μmoles). +, Addition made; −, no addition.

A

Substrate	Glucose								
Cofactors	+			−			+		
Krebs-cycle intermediates	−			+			+		
Gas phase	Air	N_2	%*	Air	N_2	%*	Air	N_2	%*
Expt. no.									
1	0·49	1·62	70	0·33	1·15	71	1·63	2·25	28
2	0·44	1·50	71	0·80†	1·70†	53	2·25	3·16	29
3	—	—	—	—	—	—	—	—	—
4	—	—	—	—	—	—	0·32	0·97	67

B

Substrate	Hexose phosphates											
Cofactors	−			+			−			+		
Krebs-cycle intermediates	−			−			+			+		
Gas phase	Air	N_2	%*	Air	N_2	%*	Air	N_2	%*	Air	N_2	%*
Expt. no.												
1‡	—	—	—	0·66	1·27	48	0·25†	0·58†	57	1·78	2·28	22
2	—	—	—	3·13	3·43	9	3·01†	3·11†	3	3·35	4·39	24
3	1·64	1·64	0	2·02	2·21	9	2·14†	2·48†	14	2·84	4·30	34
4	0·18	0·19	5	—	—	—	0·27†	0·31†	13	0·78	1·26	38

* Percentage inhibition of aerobic lactate production.
† 0·33 mM-DPN was included in the system.
‡ Glycolysis intermediate added was 0·01 M-G 6-P.

Preliminary experiments with a fortified system demonstrated that under aerobic conditions there was an accumulation of alkali-labile organic phosphate compounds. More definite evidence of the presence of hexose and triose phosphates was obtained by chromatographic separation of the perchloric acid filtrates. Data for representative experiments are shown in Table 7.

Radioautographs did not reveal any spots corresponding to glycolysis intermediates under aerobic or anaerobic conditions in the absence of added cofactors and Krebs-cycle intermediates (Expt. 1 of Table 7). With cofactors present six spots (including inorganic phosphate) were detected both under aerobic and anaerobic conditions. However, a large quantitative difference was found: all compounds were present in larger amounts in the aerobic samples, and especially two intermediates which were identified by chemical tests as well as by chromatogram as FDP and G 6-P.

These findings make it clear that the first steps of glycolysis, the phosphorylation of glucose, proceeded at a much higher rate under aerobic conditions. The fact that hexose phosphates accumulated points to the possibility of a partial block below the FDP step which prevents its breakdown.

In the following experiments the inhibition occurring below the FDP step was mainly studied.

Aldolase. First it was determined whether or not the activity of aldolase was inhibited under aerobic conditions. Aldolase activity was assayed by measuring FDP disappearance and triose phosphate formation. However, since the methods used for the estimation of these compounds were based on phosphate determinations, the concentration of inorganic phosphate chosen was lowered. It was found that when the inorganic phosphate concentration was varied from 3 to 13 mM, anaerobic glycolysis was reduced by only 10% at the lowest phosphate concentration and the aerobic inhibition of lactate production remained unchanged.

If there were a block at the aldolase step, the rate of aerobic formation of triose phosphate would be decreased. However, it was found that triose phosphate formation was not impaired

Table 7. *Accumulation of intermediates of glycolysis under aerobic conditions*

System: standard medium. Cofactors: 2 mM-ATP, 0·33 mM-DPN. Expt. 1 substrate: 3 mM-glucose. Mitochondria equivalent to 600 mg. of fresh brain were added. ^{32}P 3 600 000 counts/min./vessel (7 μC). Volume, 1·5 ml. Expt. 2 substrates: 6·7 mM-glucose, 7·6 mM-α-oxoglutarate. Mitochondria equivalent to 800 mg. of fresh brain were added, ^{32}P 8 220 000 counts/min./vessel (16 μC). Volume, 3 ml. Duration of experiments, 30 min. Glycolysis intermediates were determined by quantitative chromatography (cf. Experimental section). +, Addition of cofactors; −, no cofactors.

Gas phase		O_2 consumed (μmoles)	Glucose consumed (μmoles)		Lactate formed (μmoles)		Inhibition of aerobic lactate formation (%)	FDP formed (μmoles)		G 6-P formed (μmoles)		Other glycolysis intermediates (μmoles)	
Expt. no.	Substrates (μmoles)		Air	N_2	Air	N_2		Air	N_2	Air	N_2	Air	N_2
	Cofactors												
1	Glucose (4·5)	−	1·61	0·40	0·23	1·62	86	0	0	0	0	0	0
		+	4·22	4·16	1·43	2·02	30	0·61	0·04	0·20	(0·005)	0·26	0·12
2	Glucose (20), α-oxoglutarate (22·8)	+	20·7	11·6	5·2	6·16	8·66	29	3·57	0·26	1·08	0·26	0·70	0·60

under aerobic conditions (Fig. 2). Moreover, the steady-state concentrations of triose phosphate were not diminished by aerobic conditions (Table 9). In fact they were slightly higher than the anaerobic figures, since more FDP remained than in the anaerobic samples, where more FDP was consumed, as shown by the higher figures for lactate and PGA.

It is well known that iodoacetate in low concentrations inhibits glycolysis by blocking the triose phosphate dehydrogenase without, however, impairing to the same extent the respiratory activity. Thus by using FDP as substrate in the presence of iodoacetate, aldolase was effectively the only enzyme measured in the glycolysis reactions. At iodoacetate concentrations of 3–10 mM, the rates of formation of triose phosphates under aerobic and anaerobic conditions were identical.

These experiments showed also that the aerobic inhibition of lactate formation occurred from the start of the incubation and remained almost unchanged during the experimental period (40 min.). The aldolase activity was clearly not involved in the aerobic inhibition since the steady state was reached with equal speed under aerobic and anaerobic conditions.

Effect of fluoride. The activity of the enzymes of the glycolysis reactions, except for the enolase and enolphosphopyruvate kinase, can be determined by

Fig. 2. Activity of aldolase in the presence of fluoride and pyruvate in brain mitochondria. System: standard medium (inorganic phosphate 6 mM). Additions: 4·5 mM-FDP, 6·7 mM-α-oxoglutarate, 2 mM-AMP, 0·33 M-DPN, 10 mM-NaF, 4·5 mM-pyruvate. The reaction was started by addition of a suspension of mitochondria derived from 300 mg. of fresh brain. Total volume, 1·5 ml. Gas phase: ○, air; ●, N_2.

measuring lactate production after blocking with fluoride and adding pyruvate to reoxidize the reduced diphosphopyridine nucleotide (DPNH) formed at the glyceraldehyde phosphate dehydrogenase step (Meyerhof & Kiessling, 1935). It is evident from the results shown in Table 8 and Fig. 2 that, even after exclusion of these two enzymes, the aerobic inhibition of lactate formation persisted. At the concentration tested (0·01 M) fluoride inhibited glycolysis by about 90 % but had a much smaller effect on the respiration. The lactate formation in the presence of pyruvate and fluoride was about the same or slightly less than that observed without fluoride.

Fluoride appeared to increase the aerobic inhibition of glycolysis. With different mitochondrial preparations the percentage inhibition with fluoride, with FDP as glycolytic substrate, was a little higher [$44 \pm 2{\cdot}89$ % (7 experiments)] than without fluoride [$35 \pm 1{\cdot}74$ % (11 experiments)].

In the experiments with fluoride and pyruvate, two main reactions could be involved in the inhibi-

Table 8. *Effect of fluoride and pyruvate on respiration and glycolysis in brain mitochondria*

System: standard medium. Cofactors, 2 mM-ATP, 0·33 mM-DPN, 10 mM-NaF. Additions were as indicated in the table. Total volume, 1·5 ml. +, Addition of fluoride; −, no fluoride.

Expt. no.	Additions (μmoles)	NaF	Mitochondria (mg.)	Incubation time (min.)	O_2 consumed (μmoles)	Lactate formed (μmoles)		Inhibition (%)
						Aerobic	Anaerobic	
1	Glucose (15), pyruvate (4), L-malate (3)	−	160	35	3·41	0·66	1·26	48
	Glucose (15), pyruvate (4), L-malate (3)	+	160	35	2·17	0·78	1·15	32
2	Glucose (15), pyruvate (4), L-malate (3)	−	100	30	0·8	0·44	1·33	67
	Glucose (15), pyruvate (4), L-malate (3)	+	100	30	0·55	0·20	0·75	73
3	FDP (10), pyruvate (8)	−	220	40	—	2·82	3·46	19
	FDP (10), pyruvate (8)	+	220	40	—	2·49	3·40	26
4	FDP (12), α-oxoglutarate (12), pyruvate (10)	−	350	60	10·2	1·15	2·14	46
	FDP (12), α-oxoglutarate (12), pyruvate (10)	+	350	60	4·5	1·07	2·62	59

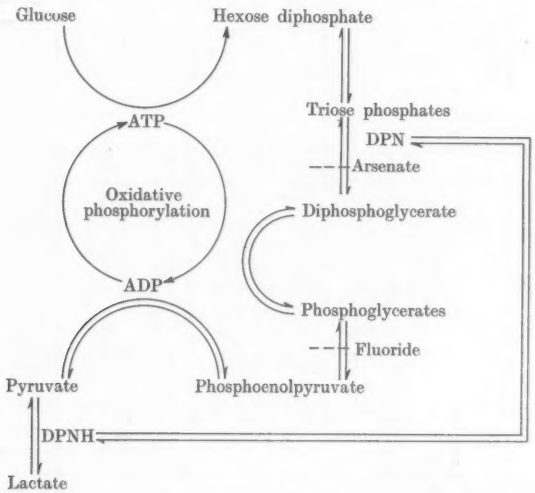


Fig. 3. Schematic representation of glycolysis, oxidative phosphorylation and site of action of inhibitors used.

Table 9. *Phosphoglycerate and lactate accumulation in brain mitochondria blocked by fluoride and supplemented with pyruvate*

System: standard medium; 0·01 M-NaF; the concentrations of inorganic phosphate and the other additions are indicated in the table. Mitochondria were added equivalent to 600 mg. of fresh brain, except in Expt. 4 where 1 g. was used. Duration of experiment, 40 min., except in Expts. 5 (*a*) and 5 (*b*) where the incubation time was 30 min. Total volume, 3 ml., except in Expts. 5 (*a*) and 5 (*b*) where the volume was 1·5 ml.

Gas phase		O_2 consumed (μmoles)	Fructose diphosphate consumed (μmoles)		Triose phosphates formed (μmoles)		Phospho-glycerate formed (μmoles)		Inhibition (%)	Lactate formed (μmoles)		Inhibition (%)
			Air	N_2	Air	N_2	Air	N_2		Air	N_2	
Expt. no.	Additions (μmoles)											
1*‖	FDP (19·2), pyruvate (24), L-malate (8·1), inorganic phosphate (18), ATP (6), DPN (0·99)	10·0	7·15	7·0	10·45	10·45	0·95	2·02	53	1·11	2·41	54
2‖	FDP (14·4), pyruvate (24), L-malate (8·1), inorganic phosphate (18·9), ADP (4·8), AMP (2·7), DPN (0·99)	19·9	5·16	6·45	8·65	8·0	2·42	4·5	46	2·96	5·5	46
3‖	FDP (14·1), pyruvate (19·8), L-malate (8·1), inorganic phosphate (17·7), AMP (6), DPN (0·99)	14·6	5·97	9·31	9·04	8·22	2·35	3·83	39	3·52	6·56	46
4†	FDP (19·5), pyruvate (19·8), α-oxo-glutarate (24), inorganic phosphate (18), ATP (6), DPN (0·99)	26·6	6·0	8·5	11·5	10·3	2·95 (1·74)	4·91 (3·26)	40 (46)	3·33	6·66	50
5 (*a*)‡	FDP (8·25), α-oxoglutarate (13), pyruvate (9·9), inorganic phosphate (9), ATP (3), DPN (0·66)	—	3·24	3·0	5·96	5·16	0·54	0·79	32	0·93	1·67	44
5 (*b*)§	α-Oxoglutarate (13), pyruvate (9·9), inorganic phosphate (9), ATP (3), DPN (0·66), 3-phosphoglycerate (8·6)	—	—	—	—	—	8·6	8·9	—	0·27	0·41	34

* Precipitation of inorganic phosphate by the method of Lipmann & Tuttle (1944).

† Phosphoglycerate was measured by chromatography, solvent ethyl acetate–acetic acid–water (4:3:1). Figures in parentheses represent the results obtained by the chromatographic method of Cowgill (1955) (the 3-phosphoglycerate only was measured).

‡ Phosphoglycerate was determined by the method of Cowgill (1955) (figures represent only the 3-phosphoglycerate).

§ Phosphoglycerate was measured in the nucleotide-free filtrate as the difference between total digestible phosphate and phosphate hydrolysed in N-HCl in 30 min. at 100°.

‖ Phosphoglycerate was determined as stable phosphate.

tion of lactate formation, after exclusion of the aldolase: (*a*) the oxidation of glyceraldehyde phosphate and (*b*) the oxidation of DPNH by lactic dehydrogenase. If the oxidation of glyceraldehyde phosphate were as rapid under aerobic as under anaerobic conditions, and if the DPNH formed at this step was partly oxidized by the electron-transport chain (as was suggested by Ball, 1939), the same amount of PGA would be formed despite the difference in the accumulated lactate. (This would apply also if the lactic dehydrogenase were inhibited.) If, on the other hand, the oxidation of triose phosphates was inhibited in the aerobic sample, the accumulation of PGA would be inhibited to the same extent as that of lactate. (These possibilities can be readily visualized in Fig. 3.) In the following experiments (fortified system, containing FDP as glycolysis substrate, fluoride and excess of pyruvate) these possibilities were tested by measuring accumulation of both lactate and PGA.

In a control experiment it was found that PGA added in the place of FDP did not disappear in the course of the incubation (Table 9, Expt. 5*b*). Thus the PGA determined represented the actual amount of this compound formed.

It was found that lactate values were higher than the corresponding PGA values, although one would have expected a very close agreement. However, it is evident from the data presented in Table 9 that the aerobic accumulation of both these compounds was inhibited to about the same extent. Moreover this was confirmed and a closer agreement was found in the absolute figures for PGA and lactate when the former was measured by the specific method of Meyerhof & Schulz (1938); (Balázs & Lagnado, 1959). These results validate the second explanation for the aerobic inhibition of lactate formation, i.e. that implicating the inhibition of the oxidation of glyceraldehyde phosphate.

Triose phosphate dehydrogenase. The oxidation of glyceraldehyde phosphate to PGA is performed by two enzymes, the dehydrogenase and the diphosphoglycerate kinase. A block at either step could account for the inhibition of the oxidation, since both these steps are reversible. The following experiments were carried out to determine which of these two enzymes was inhibited. Diphosphoglycerate kinase can be excluded by assaying triose phosphate dehydrogenase activity in the presence of arsenate, which, it is well established (Warburg & Christian, 1939*a*, *b*; Needham & Pillai, 1937), can replace phosphate to form phosphoglyceryl arsenate which is spontaneously hydrolysed to 3-phosphoglycerate. In order to make this test valid, the following had to be considered (Crane & Lipmann, 1953): (*a*) the arsenate concentration must be such that it cannot markedly affect oxidative phosphorylation (this was found to apply with the preparations used when the arsenate:inorganic phosphate ratio was 2); (*b*) arsenate does not itself block respiration but can give rise during incubation to arsenite, which is a strong inhibitor of respiration, especially in the presence of keto acids (but not with succinate). It was found that respiration was only slightly inhibited in the presence of arsenate (and remained linear during the experimental period tested), when either keto acids or succinate were used as oxidative substrate (Table 10). Thus the use of arsenate was a valid means of excluding mainly the diphosphoglycerate kinase. That this enzyme was not involved in the aerobic inhibition of lactate formation was demonstrated by the finding that arsenate did not modify the aerobic inhibition of lactate formation (Table 10).

Table 10. *Effect of arsenate on the aerobic inhibition of lactate formation in brain mitochondria*

System: standard medium (inorganic phosphate 4 mM). Substrates: 10 mM-FDP, 8·7 mM-α-oxoglutarate, 8·5 mM-succinate. Cofactors: 1·6 mM-AMP, 0·7 mM-ATP, 0·33 mM-DPN. Mitochondria were added equivalent to 250 mg. of fresh brain. Incubation time for Expt. 1, 20 min.; for Expt. 2, 30 min. Volume, 1·5 ml.

Expt. no.	Additions	Final concn. of sodium arsenate (M)	O_2 consumed (μmoles)	Lactate formed (μmoles)		Inhibition (%)
				Aerobic	Anaerobic	
1	FDP, α-oxoglutarate, cofactors	—	2·75	0·27	0·41	34
	FDP, α-oxoglutarate, cofactors	0·008	2·31	0·26	0·41	36
	FDP, succinate, cofactors	—	3·58	0·28	0·45	38
	FDP, succinate, cofactors	0·008	2·94	0·30	0·52	41
2	FDP, α-oxoglutarate, cofactors	—	2·9	0·44	0·65	32
	FDP, α-oxoglutarate, cofactors	0·008	2·5	0·51	0·70	27
	FDP, α-oxoglutarate, 10 mM-NaF, 6·6 mM-pyruvate	—	2·7	0·40	0·58	31
	FDP, α-oxoglutarate, 10 mM-NaF, 6·6 mM-pyruvate	0·008	2·8	0·60	0·85	29

Since aldolase and lactic dehydrogenase in brain mitochondria were not present in limiting amounts (Balázs & Lagnado, 1959), the measurement of lactate formation in the presence of FDP, fluoride, pyruvate and arsenate was in fact a measure of triose phosphate dehydrogenase activity.

It is concluded from these findings that, under the present experimental conditions, the aerobic inhibition of lactate formation in brain mitochondria occurs at the triose phosphate dehydrogenase step.

DISCUSSION

At first sight, the evidence obtained as to the point of the aerobic inhibition of lactate formation in brain-mitochondria preparations appears contradictory. The experiments in which different glycolytic intermediates were tested in a system fortified with cofactors pointed to an inhibition of the enzymes phosphorylating glucose, and to another partial block below the FDP step (Table 5). On the other hand, an accumulation of G 6-P and of FDP was observed in a system containing glucose and fortified with cofactors (Table 7). This finding is not consistent with an inhibition at the level of the phosphorylating enzymes. The interpretation of the results was further complicated by the finding that no aerobic inhibition of glycolysis could be observed with FDP as glycolysis substrate in an unfortified system, although, under similar conditions, the inhibition was maximal with glucose as substrate. Further, the addition of cofactors and of Krebs-cycle intermediates caused an increase in the inhibition when FDP was used as substrate [from 0–5 % to $35 \pm 2{\cdot}35$ % (11 experiments)] and a decrease with glucose [from $83 \pm 2{\cdot}5$ % (8 experiments) to $39 \pm 2{\cdot}3$ % (37 experiments)].

These apparent contradictions were resolved after it was found that triose phosphate dehydrogenase was the site of the inhibition 'below the FDP step' (Table 10).

The experiments of Needham, Siminovitch & Rapkine (1951) and Racker & Krimsky (1948) have shown that in a multi-enzyme system such as that for glycolysis, an enzyme which is not limiting as far as the utilization of its substrate is concerned may become rate-limiting in another function, e.g. in the production of energy-rich phosphate.

In the present work it was found that respiration and also oxidative phosphorylation were low in the unfortified system. Glycolytic activity in such a system is almost entirely dependent on the production of high-energy phosphates through glycolysis itself. The partial inhibition of triose phosphate dehydrogenase leads to a decreased formation of ATP, thus impairing the phosphorylation of glucose and slowing the formation of substrate for triose phosphate dehydrogenase. Therefore less ATP is formed. 'This vicious circle leads to an amplification of the inhibitory effect on overall glycolysis which is out of proportion to the effect on the single enzyme' (Racker & Krimsky, 1948).

That it is not the enzyme itself, but rather the lack of the product of its activity, which becomes rate-limiting in an earlier part of the glycolysis reactions is demonstrated by the finding that the inhibition was not observed if the phosphorylated intermediate, FDP, was used.

With a brain-mitochondria preparation, however, in a system supplemented with cofactors and Krebs-cycle intermediates, rates of both respiration and oxidative phosphorylation were high. The high ATP content maintained by oxidative phosphorylation gives rise to accumulation of G 6-P and FDP. Thus when either glucose or FDP is added to an aerobic fortified system, FDP is always present in excess, and ensures the presence of sufficient glyceraldehyde phosphate to act as substrate for triose phosphate dehydrogenase. However, because of the inhibition of this enzyme under aerobic conditions, the level of triose phosphate dehydrogenase itself becomes rate-limiting, although it was not a pace-maker in the unfortified system. These mechanisms are represented schematically in Fig. 3 as they operate under the different experimental conditions.

The aerobic inhibition of triose phosphate dehydrogenase observed in brain-mitochondria preparations would appear to invalidate some of the hypotheses put forward to explain the Pasteur effect, at least as it occurs in this type of cell-free system.

The oxidative removal of DPNH (Ball, 1939) played no major role in the investigated phenomenon. (Phosphoglycerate formation, as a measure of DPNH production at the triose phosphate dehydrogenase step, was inhibited to the same extent as the reduction of pyruvate; Table 9.)

There is no indication of an inhibition of phosphofructokinase, as was first suggested by Engelhardt & Sakov (1943) and more recently by Aisenberg & van Potter (1957). Our findings in this respect (cf. Table 7) agree with those reported by Gatt (1957).

The direct glucose-oxidation pathway does not seem to be implicated in the inhibition observed in our system since, for example, the addition of TPN had no effect on the inhibition.

The possibility of an inhibition of triose phosphate dehydrogenase was previously suggested by several investigators. There were mainly two types of interpretations given: (*a*) triose phosphate oxidation requires cofactors (ADP, DPN) and inorganic phosphate for activity, so that during oxidative phosphorylation the enzymes involved could well be deprived of inorganic phosphate and of

phosphate acceptors (Lynen, 1941; Johnson, 1941; Chance, 1956); (*b*) as was first suggested by Lipmann (1933, 1934), oxygen could have a direct 'braking effect' on glycolysis, more specifically by inhibiting triose phosphate oxidation (Lipmann, 1942). Until now, the only evidence in support of this hypothesis has been of an indirect nature and is based on the sensitivity of triose phosphate dehydrogenase to SH-reagents (Quastel & Wheatley 1932; Bumm & Appel, 1932; Wagner-Jauregg & Rzeppa, 1936; Gemill & Hellerman, 1937; Rapkine, 1938; Chaix & Fromageot, 1939).

Since arsenate can replace inorganic phosphate at the triose phosphate dehydrogenase step (Needham & Pillai, 1937; Warburg & Christian, 1939*a*, *b*) and arsenate has not influenced the inhibition, the possibility that the inhibition was mediated through the availability of inorganic phosphate seems to be excluded. Moreover, it was found that the inhibition was not dependent on the concentration of inorganic phosphate (range tested 3–13 mM).

The possibility that the inhibition of oxidation of triose phosphate is mediated through the unavailability of phosphate-acceptors was also excluded, since, under suitable experimental conditions (with FDP and Krebs-cycle intermediates as substrates; cf. Table 6), the addition of phosphate-acceptors in fact increased the inhibition. Moreover, the inhibition remained in the presence of arsenate, although in this case oxidation of triose phosphate no longer required phosphate-acceptors (see Table 10). It therefore appears that the second interpretation (Lipmann, 1942), namely a direct inhibition of glycolysis by aerobic processes, could account for the present results.

However, if the inhibition of triose phosphate dehydrogenase can explain the present results found with a brain-mitochondria preparation with which glycolysis is associated, it does not necessarily follow that it can also explain all the events which occur in the intact cell. If this were the only mechanism operative, there should be an accumulation of FDP (Krebs, 1957); this occurs in cell-free systems (Kalckar, 1939; Colowick, Kalckar & Cori, 1941; Gatt, 1957; cf. present work), but has never been observed in intact tissues. It would appear that the control of glucose metabolism by aerobiosis is not a single-step reaction and, though the aerobic inhibition of triose phosphate dehydrogenase can account for a part of the effect, it is clear that some other mechanism which prevents the phosphorylation of glucose must also be involved in the intact cell. One possible explanation of the nature of the inhibition is that the availability of ATP for the phosphorylating enzymes is limited through the 'compartmentalization' of the cell (Lynen, 1955; Hess & Chance, 1958). Another tentative explanation based on the work of Weil-Malherbe & Bone (1951), Crane & Sols (1953) and Lardy & Parks (1956) would implicate the regulation of the phosphorylating steps in glycolysis by products formed through the activity of the enzymes concerned (G 6-P), or by factors, the concentrations of which depend on the energy balance in the cell (ADP and ATP: magnesium ratio). In line with these suggestions is the idea proposed by Racker (1956) that glucose metabolism may be regulated by the shuttling of limited amounts of adenine nucleotides between the respiratory- and glycolytic-enzyme systems. However, any attempted explanation of the aerobic inhibition of glycolysis in the intact cell is speculative in the present state of our knowledge.

SUMMARY

1. Glycolytic activity associated with 'sucrose mitochondria' from rat brain is inhibited ($83 \pm 2{\cdot}5$ %) under aerobic conditions.

2. Glycolytic activity is increased by the addition of cofactors (adenosine triphosphate, adenosine diphosphate or adenosine monophosphate and diphosphopyridine nucleotide), but the aerobic inhibition of lactate formation is thereby decreased to 59 ± 4 %.

3. In the presence of cofactors, the addition of Krebs-cycle intermediates further increases glycolytic activity and reduces the aerobic inhibition to $39 \pm 2{\cdot}3$ %.

4. Glucose consumption in an unfortified system is depressed under aerobic conditions, but exceeds the anaerobic glucose consumption by two to six times in a fortified system. The glucose utilized accumulates mainly as fructose diphosphate and glucose 6-phosphate.

5. With fructose diphosphate as substrate instead of glucose, no aerobic inhibition of lactate formation is observed unless cofactors and Krebs-cycle intermediates are added, in which case a $35 \pm 1{\cdot}7$ % inhibition reappears.

6. It was demonstrated that triose phosphate dehydrogenase is the main site of the aerobic inhibition of lactate formation in brain-mitochondria preparations.

7. The results obtained can be explained on the basis of the inhibition of triose phosphate dehydrogenase. Depending upon the experimental conditions used, the depression of lactate formation is due either to the inhibition of the enzyme itself (fortified system) or indirectly to the lack of adenosine triphosphate produced at this step (unfortified system).

The competent technical assistance of Mr W. Jones and Mr R. G. Fish is acknowledged. I wish to thank Dr D. Richter for his constant interest, encouragement and useful

criticisms; Sir Hans Krebs for helpful discussions; Dr D. M. Needham for her many valuable suggestions and for great help in revising the manuscript; Dr J. Lagnado for assistance in drafting the manuscript and for the many discussions during the course of this work. A personal grant from the Rockefeller Foundation and a grant for equipment from the Medical Research Council are gratefully acknowledged.

REFERENCES

Ács, Gy., Garzó, T., Grosz, Gy., Molnár, J., Stephaneck, O. & Straub, F. B. (1955). *Acta physiol. Hung.* **8**, 269.

Aisenberg, A. C., Reinafarje, B. & van Potter, R. (1957). *J. biol. Chem.* **224**, 1099.

Aisenberg, A. C. & van Potter, R. (1957). *J. biol. Chem.* **224**, 1115.

Aldridge, W. N. (1957). *Biochem. J.* **67**, 423.

Balázs, R. & Lagnado, J. (1959). *J. Neurochem.* (in the Press).

Balázs, R. & Richter, D. (1958). *Biochem. J.* **69**, 57P.

Ball, E. G. (1939). *Johns Hopk. Hosp. Bull.* **65**, 253.

Bandurski, R. S. & Axelrod, B. (1951). *J. biol. Chem.* **193**, 405.

Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.

Benson, A. A., Bassham, J. A., Calvin, M., Godale, T. S., Haas, V. A. & Stephen, W. (1950). *J. Amer. chem. Soc.* **72**, 1740.

Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.

Birmingham, M. K. & Elliott, K. A. C. (1951). *J. biol. Chem.* **189**, 73.

Brody, T. M. & Bain, J. A. (1952). *J. biol. Chem.* **195**, 685.

Bumm, E. & Appel, H. (1932). *Hoppe Seyl. Z.* **210**, 79.

Burk, D. (1937). *Occ. Publ. Amer. Ass. Advanc. Sci.* **4**, 121.

Burk, D. (1939). *Cold Spr. Harb. Symp. quant. Biol.* **7**, 420.

Chaix, P. & Fromageot, C. (1939). *Enzymologia*, **6**, 33.

Chance, B. (1956). In *Enzymes; Units of Biological Structure and Function*, p. 447. Ed. by Gaebler, O. H. New York: Academic Press Inc.

Colowick, S. P., Kalckar, H. M. & Cori, C. F. (1941). *J. biol. Chem.* **137**, 343.

Cowgill, R. W. (1955). *Biochem. Biophys. Acta*, **14**, 614.

Crane, R. K. & Lipmann, F. (1953). *J. biol. Chem.* **201**, 235.

Crane, R. K. & Sols, A. (1953). *J. biol. Chem.* **203**, 273.

Dent, C. E. (1947). *Biochem. J.* **41**, 240.

Dickens, F. (1951). In *The Enzymes: Chemistry and Mechanism of Action*, p. 624. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.

Dixon, K. C. (1937). *Biol. Rev.* **12**, 431.

Elliott, K. A. C. & Henry, M. (1946). *J. biol. Chem.* **163**, 361.

Engelhardt, W. A. & Sakov, N. E. (1943). *Biokhimiya*, **8**, 9.

Friedemann, L. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.

Gallagher, C. H., Judah, J. O. & Rees, K. R. (1956). *Biochem. J.* **62**, 436.

Gatt, S. (1957). *Fed. Proc.* **16**, 184.

Gatt, S., Krimsky, I. & Racker, E. (1956). *Fed. Proc.* **15**, 259.

Gemill, C. C. & Hellerman, L. (1937). *Amer. J. Physiol.* **120**, 522.

Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.

Hess, B. & Chance, B. (1958). *4th Int. Congr. Biochem., Vienna*, p. 69.

Hesselbach, M. L. & Du Buy, H. G. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 62.

Johnson, N. P. (1941). *Science*, **94**, 200.

Kalckar, H. (1939). *Biochem. J.* **33**, 631.

Kiessling, W. (1938). *Biochem. Z.* **298**, 421.

Krebs, H. A. (1957). *Endeavour*, **16**, 125.

Krebs, H. A. & Johnson, W. A. (1937). *Biochem. J.* **31**, 645.

Lardy, H. A. & Parks, R. E. jun. (1956). In *Enzymes: Units of Biological Structure and Function*, p. 584. Ed. by Gaebler, O. H. New York: Academic Press Inc.

LePage, G. A. (1948). *J. biol. Chem.* **176**, 1009.

Lipmann, F. (1933). *Biochem. Z.* **265**, 133.

Lipmann, F. (1934). *Biochem. Z.* **268**, 205.

Lipmann, F. (1942). In *A Symposium on Respiratory Enzymes*, p. 48. Madison, U.S.A.: University of Wisconsin Press.

Lipmann, F. & Tuttle, L. C. (1944). *J. biol. Chem.* **153**, 571.

Lohmann, K. & Jendrassik, L. (1927). *Biochem. Z.* **178**, 419.

Lynen, F. (1941). *Liebigs Ann.* **546**, 120.

Lynen, F. (1955). *3ième Congr. Int. Biochim., Bruxelles*, p. 294.

Mendel, B., Bauch, M. & Strelitz, F. (1931). *Klin. Wschr.* **10**, 118.

Meyerhof, O. & Kiessling, W. (1935). *Biochem. Z.* **283**, 83.

Meyerhof, O. & Lohmann, K. (1934). *Biochem. Z.* **271**, 89.

Meyerhof, O. & Schulz, W. (1938). *Biochem. Z.* **297**, 60.

Mortimer, E. C. (1952). *Canad. J. Chem.* **30**, 653.

Needham, D. M. & Pillai, R. K. (1937). *Biochem. J.* **31**, 1837.

Needham, D. M., Siminovitch, L. & Rapkine, S. M. (1951). *Biochem. J.* **49**, 113.

Nelson, N. (1944). *J. biol. Chem.* **153**, 375.

Quastel, J. H. & Wheatley, A. H. M. (1932). *Biochem. J.* **26**, 2169.

Racker, E. (1956). *Ann. N.Y. Acad. Sci.* **63**, 1017.

Racker, E. & Krimsky, I. (1948). *J. biol. Chem.* **173**, 159.

Rapkine, L. (1938). *Biochem. J.* **32**, 1729.

Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.

Strecker, H. J. (1957). In *Metabolism of the Nervous System*, p. 469. Ed. by Richter, D. London: Pergamon Press.

Terner, C. (1954). *Biochem. J.* **56**, 471.

Terner, C. (1956). *Biochem. J.* **64**, 523.

Tiedemann, H. & Born, J. (1958). *Biochim. biophys. Acta*, **27**, 657.

Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism.* Minneapolis: Burgess Publishing Co.

Wagner-Jauregg, T. & Rzeppa, H. W. (1936). *Hoppe Seyl. Z.* **243**, 166.

Warburg, O. (1926). *Stoffwechsel der Tumoren.* Berlin: Springer Verlag.

Warburg, O. & Christian, W. (1939*a*). *Biochem. Z.* **301**, 221.

Warburg, O. & Christian, W. (1939*b*). *Biochem. Z.* **303**, 40.

Weil-Malherbe, H. (1937). *Biochem. Z.* **31**, 2202.

Weil-Malherbe, H. & Bone, A. D. (1951). *Biochem. Z.* **49**, 339.

Wu, R. & Racker, E. (1957). *Fed. Proc.* **16**, 274.

Biochemistry of Sheep Tissues

ENZYME SYSTEMS OF LIVER, BRAIN AND KIDNEY

By C. H. GALLAGHER and S. H. BUTTERY*
McMaster Animal Health Laboratory, Division of Animal Health and Production, C.S.I.R.O., Parramatta Road, Glebe, N.S.W. and Sheep Biology Laboratory, C.S.I.R.O., Prospect, N.S.W., Australia

(*Received* 23 *October* 1958)

Little information is available on the enzyme activities of sheep tissues *in vitro*. Kleiber (1941, 1947) measured the oxygen consumption of liver slices of rats, rabbits and sheep, and attempted to correlate the Q_{O_2} of this tissue with the metabolic rate of the living animal and to establish a direct relationship to body size. However, Krebs (1950), using more refined and standardized techniques, has shown that, although the Q_{O_2} values of the tissues of larger animals are lower than the corresponding values for smaller species, there is no direct relationship to the basal heat production of the intact animal. The Q_{O_2} value for most tissues changes much less with body weight than does the rate of heat production. Krebs attributed the differences in basal heat production in animals of different size mainly to variation in the Q_{O_2} of muscle, and suggests that the metabolic rate of other tissues is governed by the specific energy requirements of the particular tissue and not by the heat requirements of the whole body. Comparative Q_{O_2} values from various tissues and species, including sheep, are published by Krebs (1950) and Albritton (1954). These figures are of general interest but cannot supply information on the activities of isolated enzyme systems. More specific data are available on the metabolism of short-chain fatty acids by sheep-rumen epithelium and slices of kidney and liver (Pennington, 1952, 1954; Annison & Pennington, 1954; Pennington & Sutherland, 1954, 1955, 1956*a*), ketone-body production from certain substrates by rumen epithelium (Pennington & Sutherland, 1956*b*) and the metabolism of acetate and glucose by sheep mammary-gland slices, particularly in relation to lipogenesis (Folley & French, 1950; Balmain, Folley & Glascock, 1952, 1954; Duncombe & Glascock, 1953, 1956).

The aims of the present study were: to measure the activity of enzyme systems of sheep liver, brain and kidney; to observe where and how the metabolic pattern differs from that of other species; to note variations which might be attributable to the age, sex or nutritional history; and to establish normal reference data for studies in chemical pathology.

* Present address: Animal Health Research Laboratory, C.S.I.R.O., Parkville, Victoria, Australia.

METHODS

Enzyme preparations

Sheep were bled to death by severing the cervical vessels. Large samples, at least 10 g., were selected from the same area of liver parenchyma, cerebral cortex and kidney cortex in each animal. Tissues were removed rapidly and immersed in ice-cold 0·25 M-sucrose solution. Homogenates were prepared in 0·25 M-sucrose with glass homogenizers and fractionated by differential centrifuging in the cold (Schneider, 1948). Mitochondria were re-suspended in 0·25 M-sucrose unless otherwise indicated.

Hexokinase was prepared from yeast by the method of Berger, Slein, Colowick & Cori (1946) as modified by Cross, Taggart, Covo & Green (1949). The fraction precipitating between 25 and 40% (v/v) of ethanol was dissolved in 1% glucose solution and stored frozen. The optimum addition for oxidative phosphorylation and glycolysis was determined by assay with rat enzymes.

Reagents

Adenosine triphosphate (ATP), adenosine 5′-monophosphate (AMP), coenzyme I (Co I), coenzyme II (Co II), coenzyme A (CoA), nicotinamide, reduced glutathione (GSH), glucose, glucose 1-phosphate, glucose 6-phosphate, fructose 1:6-diphosphate (HDP), pyruvate, citrate, α-oxoglutarate, L-glutamate, succinate, fumarate, L-malate, oxaloacetate, acetate, propionate, butyrate, DL-β-hydroxybutyrate, octanoate, palmitate, L-ascorbic acid and inorganic reagents were obtained commercially. Cytochrome *c* was prepared by the method of Keilin & Hartree (1937) but dialysed against distilled water; it was also obtained commercially. New reagents were checked for inhibitory impurities in the reactions for which they were to be used, rat-tissue enzyme preparations for which the reaction rates are well established being employed. Inhibitory reagents were identified by elimination and substitution and were purified as follows: AMP was recrystallized twice from hot water. Cytochrome *c* (the commercial product) was dialysed at 0–1° for 24 hr. against three changes of distilled water and standardized by the method of Potter (1957). Pyruvic acid was redistilled *in vacuo* and crystallized as described by Lardy (1957). Succinic acid was recrystallized three times from hot water, then dissolved, neutralized and precipitated as the sodium salt with ethanol, and the salt was crystallized from 80% ethanol. Octanoic acid was

Table 1. *Anaerobic glycolysis by liver and brain homogenates*

System: ATP mM; AMP mM; KCl 25 mM; $MgSO_4$ 6·7 mM; sodium potassium phosphate buffer, pH 7·4, 13·3 mM; sodium pyruvate 6·7 mM; $KHCO_3$ 0·02 M; NaF 0·01 M; Co I 0·5 mM; nicotinamide 0·04 M; GSH 0·67 mM; enzyme equivalent to 80–100 mg. of fresh tissue in 0·25 ml. of 0·25 M-sucrose; water to 1·5 ml. final vol.; gas phase $N_2 + CO_2$ (95:5); temp. 38°; equilibration period 10 min. S.D., Standard deviation.

	$Q^{N_2}_{CO_2}$ (μl. of CO_2 evolved/mg. fat-free dry wt. of enzyme/hr.)							
	Liver				Brain			
Additions	No. of animals	Range	Mean	S.D.	No. of animals	Range	Mean	S.D.
None	15	7–20	14	±4	18	4–14	9	±3
HDP (0·01 M)	15	23–51	36	±9	18	37–76	54	±12
HDP (mM)	15	12–26	19	±4	18	14–31	22	±5
Glucose (0·01 M)	15	6–17	12	±3	—	—	—	—
HDP (mM) + glucose (0·01 M)	15	10–22	17	±4	18	47–107	76	±17
None	1	17	—	—	—	—	—	—
HDP (mM)	1	21	—	—	—	—	—	—
HDP (mM) + glucose 1-phosphate (0·01 M)	1	75	—	—	—	—	—	—
HDP (mM) + glucose 6-phosphate (0·01 M)	1	74	—	—	—	—	—	—
HDP (mM) + fructose (0·01 M)	1	24	—	—	—	—	—	—

purified by distillation *in vacuo*. Sodium pyruvate, α-oxoglutaric acid and L-ascorbic acid were dissolved and neutralized with dilute NaOH just before use. Other substrates were neutralized and stored frozen as sodium salts for 3–4 weeks. Sodium potassium phosphate buffer, pH 7·4, 0·1 M, was prepared by the method of Umbreit (1957).

Analytical methods

Oxygen uptake and CO_2 evolution were measured by Warburg manometers. Unless specified the standard system used for respiratory experiments was: AMP mM; $MgSO_4$ 6·7 mM; KCl 25 mM; sodium potassium phosphate buffer, pH 7·4, 13·3 mM; cytochrome *c* 10 μM; substrate 0·01 M; enzyme in 0·5 ml. of 0·25 M-sucrose; water to 3 ml. final volume; 0·1 ml. of 20% KOH was placed in the centre well to absorb CO_2; gas phase, air; temperature, 38°; equilibration period, 10 min.

Respiratory and glycolytic activities are expressed without correction for endogenous rates in the absence of added substrate unless otherwise indicated.

Nitrogen was determined by the micro-Kjeldahl method of McKenzie & Wallace (1954), inorganic phosphate by the method of Fiske & Subbarow (1925) and glucose by the method of Somogyi (1952).

Dry weights were determined after extracting the sample three times with boiling ethanol–diethyl ether (3:1, v/v) to remove lipids, drying in an oven at 100° for 24 hr. and cooling in a desiccator.

RESULTS

The data reported below are the pooled results of experiments on all sheep studied, except where otherwise indicated.

Anaerobic glycolysis

Table 1 shows that brain and liver homogenates glycolyse HDP well but that although brain catabolizes glucose very actively, liver preparations apparently do not use this substrate. It was found that liver can glycolyse the phosphorylated sugars, glucose 1-phosphate and glucose 6-phosphate, but not fructose (Table 1).

Table 2. *Effect of hexokinase on anaerobic glycolysis by liver*

System: as in Table 1; hexokinase in 1% glucose was added at 0·02 ml./flask. Supernatant enzyme was prepared from homogenates in 0·25 M-sucrose by centrifuging at 9000 *g* for 20 min. Results are the means of three experiments.

	$Q^{N_2}_{CO_2}$ (μl. of CO_2 evolved/mg. fat-free dry wt. of enzyme/hr.)	
Additions	Homogenate	Supernatant
None	—	15
Hexokinase	—	25
HDP (mM)	20	31
HDP (mM) + hexokinase	30	46
Glucose (0·01 M)	—	11
Glucose (0·01 M) + hexokinase	—	26
HDP (mM) + glucose (0·01 M)	19	18
HDP (mM) + glucose (0·01 M) + hexokinase	41	58

These results suggest that the inability to use glucose is associated with lack of phosphorylation by glucokinase. This is confirmed in Tables 2 and 3, where the addition of yeast hexokinase enables liver enzymes to metabolize glucose.

It will be noticed in Table 2 that hexokinase also stimulates to a lesser extent the $Q^{N_2}_{CO_2}$ of mM-HDP. This is explained by the fact that glucose is added, 1·2 μmoles with the hexokinase and about 1·2 μmoles with the enzyme preparation, to give a final concentration of at least 1·5 mM, as a variable amount

of glucose would presumably also be produced during incubation from glycogen in the enzyme preparation.

Anaerobic glycolysis by liver homogenates of two new-born lambs was studied. Neither preparation used glucose at a measurable rate. Thus the deficiency in glucose utilization by sheep-liver homogenates is present from birth.

Oxidative metabolism

Glucose oxidation by liver. Liver mitochondria oxidize glucose, as shown in Table 3, but only upon the addition of hexokinase. The stimulatory effect of Co II suggests that part at least of the oxidation occurs via glucose 6-phosphate dehydrogenase and the phosphogluconate shunt.

Oxidation of tricarboxylic acid cycle and related intermediates. The rates of oxidation of a variety of metabolic substrates by homogenate and mitochondrial preparations are shown in Tables 4 and 5. Controls without substrates were carried out in about half of the mitochondrial experiments reported in Table 5. However, the rate of endogenous oxidation was so low that these controls were discontinued. Endogenous oxidations were higher for brain than liver mitochondria but still negligible. The studies of Christie, Judah & Rees (1953) have shown that brain mitochondria do not metabolize well *in vitro* unless supplemented by respiratory cofactors. Consequently the reaction mixtures used in the present study to assay the brain mitochondrial activities have been supplemented with diphosphopyridine nucleotide (DPN), nicotinamide, GSH and ATP, as indicated in Table 5. In general, the Q_{O_2} values range from one-third to one-half of the values reported for rat-liver and -brain enzymes (Christie & Judah, 1953; Christie *et al.* 1953; Gallagher, Judah & Rees, 1956*a*, *b*).

Oxidation of fatty acids. Addition of fatty acids to homogenates of sheep liver, brain and kidney does not cause an increase in oxygen uptake over the rate resulting from the oxidation of endogenous substrates and 0·67 mM-L-malate, added as a 'primer'. However, compared with mitochondria, tissue homogenates have a high rate of endogenous oxidation which may mask the oxidation of an exogenous substrate. It was observed, too, that the addition of 0·67 mM-L-malate increased greatly the oxygen uptake of liver and kidney homogenates. This suggested that L-malate was 'sparking' the oxidation of some components of the homogenates, probably in large part fatty acids. It is thus possible that the enzymes of the fatty acid oxidation chain are saturated or nearly so with endogenous substrate in sheep-liver and -kidney homogenates and so the addition of more fatty acid either does not affect the rate of oxygen consumption or depresses it by raising the fatty acid concentration to inhibitory levels. In these studies exogenous fatty acids did in fact lower endogenous oxygen uptake.

Table 3. *Oxidation of glucose by liver mitochondria*

System: ATP mM; AMP mM; $MgSO_4$ 6·7 mM; KCl 25 mM; sodium potassium phosphate buffer, pH 7·4, 13·3 mM; cytochrome *c* 10 μM; Co I 0·5 mM; nicotinamide 0·04 M; GSH 0·67 mM; mitochondria equivalent to 150 mg. of fresh tissue in 0·25 ml. of 0·25 M-sucrose; water to 1·5 ml. final vol.; gas phase, air; temp. 38°; equilibration period, 10 min. Q_{O_2} determination is based upon oxygen consumption during 35 min. after equilibration. Results are the means of two experiments.

Additions	$Q_{O_2}^{(N)}$ (μl. of O_2 taken up/mg. of enzyme N/hr.)
None	46
Hexokinase (in 0·02 ml. of 1% glucose)	69
Hexokinase + Co II (0·25 mM)	76
Glucose (0·01 M)	45
Glucose (0·01 M) + hexokinase	86
Glucose (0·01 M) + hexokinase + Co II	103

On the other hand, mitochondria of both liver and kidney oxidize fatty acids actively (Tables 6 and 7). As with other species, brain mitochondria apparently do not catalyse fatty acid oxidation. However, by analogy with the rat (Vignais, Gallagher & Zabin, 1958), it is probable that sheep brain does oxidize fatty acids, but to a degree detectable only by isotope techniques.

The system used for fatty acid oxidation differs from that for other oxidations in one important respect. The concentration of KCl necessary for optimum oxidation of fatty acids is 0·05 M, twice as much as for other substrates. Where mitochondria are added in 0·15 M-KCl, the necessary adjustment is made to give a final concentration of 0·05 M-KCl. Octanoate and shorter-chain fatty acids are oxidized at the same rate whether the mitochondria are re-suspended in 0·25 M-sucrose or 0·15 M-KCl, but palmitate oxidation is facilitated by suspension in 0·15 M-KCl.

Palmitate, octanoate, butyrate and propionate are oxidized well by liver mitochondria but β-hydroxybutyrate and acetate are oxidized poorly. The feeble oxidation of the ketone body, β-hydroxybutyrate, is in contrast with the brisk oxidation of this substrate by enzyme preparations of rat liver (Christie & Judah, 1954). Sheep-kidney mitochondria, on the other hand (Table 7), oxidize acetate and octanoate well but have a rather poor rate of oxidation for propionate.

Oxidative phosphorylation. The efficiency of oxidative phosphorylation by sheep-mitochondrial preparations (Table 8) is of the order found with mitochondria of other species in the same reaction mixture.

Table 4. *Oxidative metabolism of tissue homogenates*

System: standard; enzyme equivalent to 50 mg. of fresh liver and 100 mg. of fresh brain was added in 0·5 ml. of 0·25 M-sucrose, except for succinate oxidation in which the equivalents of 25 mg. and 50 mg. were added respectively. Q_{O_2} calculations are based upon the oxygen consumption during 20 min. after equilibration.

Q_{O_2} (μl. of O_2 taken up/mg. fat-free dry wt. of enzyme/hr.)

	Liver				Brain			
Additions	No. of animals	Range	Mean	S.D.	No. of animals	Range	Mean	S.D.
None	16	4–9	6	±1	10	8–25	17	±7
Citrate (0·01 M)	12	10–26	18	±6	7	7–25	17	±7
α-Oxoglutarate (0·01 M)	11	11–24	15	±4	5	24–33	28	±3
Succinate (33 mM)	12	22–74	43	±18	9	31–48	41	±6
Fumarate (0·01 M)	5	13–25	19	±5	3	20–25	22	±3
L-Malate (0·01 M)	8	14–23	18	±3	6	27–35	32	±3
Oxaloacetate (0·01 M) + pyruvate (0·67 mM)	5	12–22	17	±4	—	—	—	—
Pyruvate (0·01 M) + L-malate (0·67 M)	6	10–15	13	±2	6	19–31	23	±5
L-Glutamate (0·01 M)	12	10–25	15	±4	5	27–37	32	±4

Table 5. *Oxidative metabolism of mitochondria*

System: standard for liver; standard + ATP mM; Co I 0·5 mM; nicotinamide 0·04 M; GSH 0·67 mM for brain. Mitochondria equivalent to 100 mg. of fresh liver and 200 mg. of fresh brain were added in 0·5 ml. of 0·25 M-sucrose, except for succinate oxidation in which the equivalents of 50 mg. and 100 mg. were added respectively. Q_{O_2} calculations are based upon the oxygen consumption during 20 min. after equilibration.

$Q^{(N)}_{O_2}$ (μl. of O_2 taken up/mg. of enzyme N/hr.)

	Liver				Brain			
Additions	No. of animals	Range	Mean	S.D.	No. of animals	Range	Mean	S.D.
Citrate (0·01 M)	10	47–100	78	±20	9	48–69	57	±7
α-Oxoglutarate (0·01 M)	10	99–180	129	±29	9	128–203	156	±27
Succinate (33 mM)	10	198–455	278	±79	9	192–285	233	±25
Fumarate (0·01 M)	2	89–103	96	±10	2	80–103	92	±16
L-Malate (0·01 M)	9	77–120	101	±16	9	128–183	157	±20
Pyruvate (0·01 M) + L-malate (0·67 mM)	8	97–150	132	±17	3	136–168	152	±16
L-Glutamate (0·01 M)	9	81–180	130	±34	6	166–211	183	±16

Table 6. *Oxidation of fatty acids by liver mitochondria*

System: AMP mM; ATP mM; KCl 0·05 M; $MgSO_4$ 6·7 mM; sodium potassium phosphate buffer, pH 7·4, 13·3 mM; cytochrome *c* 10 μM; $KHCO_3$, for propionate only, 6·7 mM; L-malate 0·67 mM; mitochondria equivalent to 200 mg. of fresh liver in 0·5 ml. of 0·25 M-sucrose and, for palmitate only, in 0·5 ml. of 0·15 M-KCl; water to 3 ml. final vol.; gas phase, air; temp. 38°; equilibration period, 10 min. Q_{O_2} determination is based upon oxygen uptake during 20 min.

$Q^{(N)}_{O_2}$ (μl. of O_2 taken up/mg. of enzyme N/hr.)

					Net fatty acid and L-malate oxidation		
Additions	No. of animals	Range	Mean	S.D.	Range	Mean	S.D.
None	4	55–97	72	±19	—	—	—
Palmitate (0·25 mM)	4	98–152	118	±25	41–55	46	±6
None	19	46–121	70	±19	—	—	—
Octanoate (1·67 mM)	19	104–228	140	±26	44–107	70	±15
None	4	46–103	66	±26	—	—	—
Butyrate (3·3 mM)	4	125–239	166	±50	70–136	100	±27
None	3	55–71	62	±8	—	—	—
DL-β-Hydroxybutyrate (0·01 M)	3	65–82	73	±8	10–13	11	±2
None	8	53–103	67	±17	—	—	—
Propionate (3·3 mM)	8	121–223	166	±33	68–137	99	±25
None	3	53–69	60	±8	—	—	—
Acetate (0·01 M)	3	67–80	74	±7	11–17	14	±3

Table 7. *Oxidation of fatty acids by kidney mitochondria*

System: as in Table 6; mitochondria equivalent to 200 mg. of fresh kidney in 0·5 ml. of 0·25 M-sucrose.

Additions	No. of animals	$Q_{O_2}^{(N)}$ (μl. of O_2 taken up/mg. of enzyme N/hr.) Range	Mean	Net fatty acid and L-malate oxidation
None	2	150–160	155	—
Octanoate (1·67 mM)	2	246–377	312	157
Propionate (3·3 mM)	2	161–206	184	29
Acetate (0·01 M)	2	215–306	261	106

Table 8. *Oxidative phosphorylation by liver and brain mitochondria*

System: standard + L-glutamate 0·01 M; glucose 33 mM; hexokinase in 0·02 ml. of 1% glucose; NaF 0·01 M; plus, for brain alone, ATP mM; Co I 0·5 mM; nicotinamide 0·04 M; GSH 0·67 mM. Mitochondria equivalent to 200 mg. of fresh tissue in 0·5 ml. of 0·25 M-sucrose; duration of experiment, 30 min., after 10 min. equilibration. Reaction was stopped by addition of trichloroacetic acid to 8% final concn.

Tissue	No. of expts.	P/O (μg. atoms of P esterified/μg. atom of O_2 consumed)	S.D.
Liver	7	2·4	±0·4
Brain	5	2·1	±0·3

Table 9. *Cytochrome oxidase activity of liver and brain*

System: sodium potassium phosphate buffer, pH 7·4, 33 mM; cytochrome *c* 80 μM; $AlCl_3$ 0·4 mM; sodium ascorbate, pH 7·0, neutralized and added just before start of experiment, 11·4 mM; liver equivalent to 8 mg. of fresh tissue was added as 2% homogenate in water; brain equivalent to 14 mg. of fresh tissue was added as 4% homogenate in water; water to 3 ml. final vol.; gas phase, air; temp. 38°; equilibration period 10 min. Q_{O_2} determination is based upon oxygen uptake during 20 min. and corrected for auto-oxidation of ascorbate.

Tissue	No. of animals	Q_{O_2} (μl. of O_2 taken up/mg. fat-free dry wt. of enzyme/hr.) Range	Mean	S.D.
Liver (adult)	8	194–290	249	±34
Brain (adult)	4	107–162	137	±23
Liver (lamb)	2	144–146	145	—

Specific enzyme systems

Cytochrome oxidase. Sheep liver and brain, in common with tissues of other species, have a large reserve of cytochrome oxidase activity (Table 9). The Q_{O_2} values are about half those for rat tissues and, as in the rat, the activity of this enzyme system is lower in the young than in the adult animal (Gallagher, 1955; Gallagher *et al.* 1956*b*).

Catalase. Ox liver is used preparatively as a rich source of catalase (Colowick & Kaplan, 1955), so it is not unexpected that ruminant liver should possess high catalase activity (Table 10). What is surprising is the disproportionately high activity of liver catalase as compared with other enzyme systems and other species. In general sheep enzyme systems are about one-third to one-half as active as rat enzyme systems (Gallagher, 1955; Gallagher *et al.* 1956*b*). By contrast sheep liver has about 20 times the catalase activity of rat liver. Sheep brain has very little, if any, catalase.

DISCUSSION

Sheep tissues possess quite high enzymic activities—in general about one-third to one-half of the activities of rat tissues. The ratio between the sheep and the rat for specific enzyme systems and substrate oxidations found in this study agrees with Krebs's (1950) figures for a more general system.

Within the limited number of animals studied no apparent effect of age, sex or nutrition was shown except a lower Q_{O_2} for cytochrome oxidase in the lamb than in the adult. A similar influence of age on the cytochrome oxidase system in the rat had been observed earlier (Gallagher, 1955; Gallagher *et al.* 1956*b*).

Points of interest are the high oxidative capacity of brain, with the notable exception of citrate oxidation, the rate of oxidation of L-malate and the distribution of enzymes as indicated by the relative Q_{O_2} values for homogenates and mitochondria.

It should be remembered when comparing the results for liver and brain that the brain mitochondria have an additional supplement of respiratory cofactors. However, the same medium is used for both tissues in the homogenate experiments, and these provide an adequate basis for comparison. Krebs (1950) recorded higher Q_{O_2} values for brain slices than for liver slices in a number of species, including sheep and rats, although Albritton (1954) published data showing the rat liver to be much more active than rat brain in oxidizing succinate. The present study supports

Table 10. *Catalase activity of liver and brain*

System: H_2O_2, 0·02 N, 35 ml.; sodium phosphate buffer, pH 6·8, 0·66 M, 10 ml.; enzyme, 1 ml. of 0·1% and 0·05% liver or 1 ml. of 4% and 2% brain homogenates in water; water to 50 ml. final vol.; temp. 0°; incubation period of 2 min. was terminated by discharging 5 ml. of reaction mixture into 10 ml. of N-H_2SO_4. Residual H_2O_2 was titrated with 2 mN-$KMnO_4$. Allowance was made for reducing substances in the enzyme preparation by blank incubation and titration without H_2O_2. Catalase activity is expressed as Kat.f. (Euler & Josephson, 1927), which is K/g. fat-free dry wt. of homogenate where $K = 1/t \ln (Co/Ct)$; t = time of reaction (min.); Co = concn. of H_2O_2 before addition of enzyme and Ct = concn. of H_2O_2 at time t.

Tissue	No. of animals	Kat.f. Range	Kat.f. Mean	Kat.f. S.D.
Liver	7	2200–3300	2725	±387
Brain	5	6–41	13	± 15

Krebs's findings and reveals a higher rate of oxidation by brain than by liver preparations for all substrates except succinate and citrate. Succinate is oxidized about equally fast by both tissues. The very slow oxidation of citrate by brain is in agreement with the studies of Coxon, Liébecq & Peters (1949) for pigeon brain, and Christie *et al.* (1953) and Gallagher *et al.* (1956*a*) for rat brain.

Comparing the rates of substrate oxidations by sheep brain with the rates reported by Christie *et al.* (1953) for rat brain it is apparent that L-malate is oxidized more rapidly relative to other substrates in sheep than in rat brain.

Conclusions cannot be drawn about the intracellular distribution of enzymes from these experiments. Homogenate activities are expressed on a fat-free dry-weight basis, whereas mitochondrial activities are expressed in terms of nitrogen and are therefore not strictly comparable. However, it is of interest to observe the trends shown. A good proportion of the homogenate activity for α-oxoglutarate, succinate, pyruvate and L-glutamate oxidation is present in the mitochondrial preparations. However, the rate of oxidation by mitochondria as compared with homogenates is low for citrate, fumarate and L-malate, although brain mitochondria oxidize L-malate at a faster rate compared with homogenates than do liver mitochondria. These results suggest that either a larger proportion of the enzyme system concerned is extra-mitochondrial or is lost from the mitochondria during isolation. The same pattern of enzyme distribution for rat liver was reported by Christie & Judah (1953), who found L-glutamic dehydrogenase to be entirely mitochondrial and L-malic dehydrogenase to be distributed about equally between mitochondria and supernatant.

The outstanding enzyme differences found in sheep as compared with non-ruminants are the extraordinarily high activity of sheep-liver catalase and the dependence of sheep-liver metabolism upon the oxidation of fatty acids.

The metabolic significance of catalase in the sheep is unknown but recent work suggests a possible explanation for the high activity in liver. The administration of hydrocortisone or cortisone depresses catalase activity considerably, indicating a possible regulatory role for the steroids (Troop, 1958). Dr R. L. Reid and his colleagues at the Sheep Biology Laboratory, C.S.I.R.O. (personal communication) have found the level of adrenal steroids in the plasma of normal sheep to be very low. It is, then, conceivable that the high activity of liver catalase in sheep develops as a consequence of the absence of regulatory levels of adrenocortical steroid usual in non-ruminant species. Brain, however, has hardly any catalase. Elliott (1934) reported the absence of catalase in rabbit brain.

Sheep-liver enzyme preparations cannot metabolize glucose *in vitro* unless hexokinase is added. Inability of liver homogenates to catabolize glucose anaerobically is not peculiar to sheep. Stoesz & LePage (1949) have found the same for rats and Long (1952) has shown that hexokinase activity is very low in rat-liver homogenates. However, in sheep as opposed to non-ruminants, owing to the unavailability of carbohydrate for absorption as such from the alimentary canal and the dependence upon liver gluconeogenesis to supply blood glucose and liver glycogen, failure to catabolize glucose may assume greater importance.

Glucose is not normally present in the ruminant diet and it is improbable that when present much reaches the small intestine or is absorbed from the rumen (Phillipson & McAnnally, 1942; Elsden, 1945). Even on rations very high in carbohydrate the amount which escapes ruminal fermentation and passes into the abomasum is insignificant (Weller & Gray, 1954). Large quantities of volatile fatty acids, chiefly acetic acid, propionic acid and butyric acid, are produced by fermentation of foodstuffs in the rumen and are absorbed directly from the fore-stomachs (Elsden & Phillipson, 1948). The supply of glucose for utilization by peripheral tissues, notably the central nervous system and, in the pregnant ewe, the placenta for conversion into fructose to meet foetal demands, must come from gluconeogenesis. The liver will, of course, be the major site of glucose production from non-carbohydrate sources. Consequently, it is not surprising that liver metabolism is biased towards the formation of glucose rather than its destruction. The glucokinase reaction is the most effective point for such bias to be applied. Deficiency of glucokinase, although limiting the phosphorylation of glucose,

does not much affect the reverse reaction, which is catalyzed by a different enzyme, glucose 6-phosphatase. The defect in glucose catabolism is present from birth.

Sheep liver has then an unusual pattern of metabolism, being very largely dependent upon the oxidation of fatty acids for the maintenance of tricarboxylic acid cycle activity. Under normal conditions volatile fatty acids are supplied in abundance via the portal circulation (McAnnally & Phillipson, 1942; Barcroft, McAnnally & Phillipson, 1944 and Elsden & Phillipson, 1948). Acetyl-coenzyme A is supplied by the oxidation of all fatty acids except propionic acid, which provides a source of oxaloacetate either via pyruvate and triphosphopyridine nucleotide malic dehydrogenase or, much more likely, via methylmalonyl-coenzyme A, to enter the tricarboxylic acid cycle as succinate (Lardy & Adler, 1956; Pennington & Sutherland, 1956*b*; Flavin & Ochoa, 1957). Amino acid metabolism can also provide oxaloacetate. This metabolic scheme is undoubtedly an efficient one under normal conditions. However, the unavailability of glucose, as an alternative source to fatty acids of acetyl-coenzyme A, leaves the sheep, and probably other ruminants, particularly prone to liver failure. Inhibition of the fatty acid-oxidation sequence or a fall in the supply of fatty acids to the liver will lead to a directly proportional failure of liver metabolism. Liver glycogen in the sheep will be formed essentially from non-carbohydrate sources and, if fatty acid oxidation fails, the glycogen reserve will be soon exhausted in supplying blood glucose or acetyl-coenzyme A. The glycogenic amino acids which may be available cannot much affect the issue. The possibility arises that this unusual liver metabolism with its dependence upon only one of the two usual sources of energy may be concerned in the development of certain metabolic diseases of ruminants.

SUMMARY

1. The activities of a number of enzyme systems of sheep liver, brain and kidney have been investigated.

2. Sheep tissues in general have one-third to one-half of the enzymic activities reported for rat tissues.

3. High liver catalase is the outstanding feature found in sheep.

4. Young sheep have lower cytochrome oxidase activity than adults.

5. Liver enzyme preparations are unable to catabolize glucose owing to glucokinase deficiency. The possible significance of this in relation to ruminant metabolism is discussed.

6. The dependence of sheep-liver metabolism upon the oxidation of fatty acids is suggested as a factor of possible importance in ruminant diseases.

We wish to acknowledge gratefully the skilled technical assistance of Miss Theda Flint-Gallé and Miss Diana Mann.

REFERENCES

Albritton, E. C. (1954). *Standard Values in Nutrition and Metabolism*, p. 224. Philadelphia: W. B. Saunders and Co.

Annison, E. F. & Pennington, R. J. (1954). *Biochem. J.* **57**, 685.

Balmain, J. H., Folley, S. J. & Glascock, R. F. (1952). *Biochem. J.* **52**, 301.

Balmain, J. H., Folley, S. J. & Glascock, R. F. (1954). *Biochem. J.* **56**, 234.

Barcroft, J., McAnnally, R. A. & Phillipson, A. T. (1944). *J. exp. Biol.* **20**, 120.

Berger, L., Slein, N. W., Colowick, S. P. & Cori, C. F. (1946). *J. gen. Physiol.* **29**, 379.

Christie, G. S. & Judah, J. D. (1953). *Proc. Roy. Soc.* B, **141**, 420.

Christie, G. S. & Judah, J. D. (1954). *Proc. Roy. Soc.* B, **142**, 241.

Christie, G. S., Judah, J. D. & Rees K. R. (1953). *Proc. Roy. Soc.* B, **141**, 523.

Colowick, S. P. & Kaplan, N. O. (1955). *Methods in Enzymology*, vol. 2, p. 775. New York: Academic Press Inc.

Coxon, R. V., Liébecq, C. & Peters, R. A. (1949). *Biochem. J.* **45**, 320.

Cross, R. J., Taggart, J. V., Covo, G. A. & Green, D. E. (1949). *J. biol. Chem.* **177**, 655.

Duncombe, W. G. & Glascock, R. F. (1953). *Biochem. J.* **55**, xxiii.

Duncombe, W. G. & Glascock, R. F. (1956). *Biochem. J.* **63**, 332.

Elliott, K. A. C. (1934). *Biochem. J.* **28**, 1911.

Elsden, S. R. (1945). *J. exp. Biol.* **22**, 51.

Elsden, S. R. & Phillipson, A. T. (1948). *Annu. Rev. Biochem.* **17**, 705.

Euler, H. von & Josephson, K. (1927). *Liebigs Ann.* **452**, 158.

Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.

Flavin, M. & Ochoa, S. (1957). *J. biol. Chem.* **229**, 965.

Folley, S. J. & French, T. H. (1950). *Biochem. J.* **46**, 465.

Gallagher, C. H. (1955). Ph.D. Thesis: University of London.

Gallagher, C. H., Judah, J. D. & Rees, K. R. (1956*a*). *Biochem. J.* **62**, 436.

Gallagher, C. H., Judah, J. D. & Rees, K. R. (1956*b*). *Proc. Roy. Soc.* B, **145**, 134.

Keilin, D. & Hartree, E. F. (1937). *Proc. Roy. Soc.* B, **122**, 298.

Kleiber, M. (1941). *Proc. Soc. exp. Biol., N.Y.*, **48**, 419.

Kleiber, M. (1947). *Physiol. Rev.* **27**, 511.

Krebs, H. A. (1950). *Biochim. biophys. Acta*, **4**, 249.

Lardy, H. A. (1957). *Manometric Techniques*, 3rd ed., p. 295. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minnesota: Burgess Publishing Co.

Lardy, H. A. & Adler, J. (1956). *J. biol. Chem.* **219**, 933.
Long, C. (1952). *Biochem. J.* **50**, 407.
McAnnally, R. A. & Phillipson, A. T. (1942). *J. Physiol.* **101**, 14.
McKenzie, H. A. & Wallace, H. S. (1954). *Aust. J. Chem.* **7**, 55.
Pennington, R. J. (1952). *Biochem. J.* **51**, 251.
Pennington, R. J. (1954). *Biochem. J.* **56**, 410.
Pennington, R. J. & Sutherland, T. M. (1954). *Biochem. J.* **58**, vii.
Pennington, R. J. & Sutherland, T. M. (1955). *Biochem. J.* **60**, xxxvii.
Pennington, R. J. & Sutherland, T. M. (1956*a*). *Biochem. J.* **63**, 353.
Pennington, R. J. & Sutherland, T. M. (1956*b*). *Biochem. J.* **63**, 618.
Phillipson, A. T. & McAnnally, R. A. (1942). *J. exp. Biol.* **19**, 199.
Potter, V. R. (1957). *Manometric Techniques*, 3rd ed., p. 301. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minnesota: Burgess Publishing Co.
Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.
Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
Stoesz, P. A. & LePage, G. A. (1949). *J. biol. Chem.* **180**, 587.
Troop, R. C. (1958). *Endocrinology*, **62**, 385.
Umbreit, W. W. (1957). *Manometric Techniques*, 3rd ed., p. 168. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minnesota: Burgess Publishing Co.
Vignais, P. M., Gallagher, C. H. & Zabin, I. (1958). *J. Neurochem.* **2**, 283.
Weller, R. A. & Gray, F. V. J. (1954). *J. exp. Biol.* **31**, 40.

The Occurrence of Guanidinoacetic Acid and Other Substituted Guanidines in Mammalian Liver

By H. ROSENBERG
Department of Biochemistry, The John Curtin School of Medical Research, Australian National University, Canberra, A.C.T.

(*Received* 23 *December* 1958)

The synthesis of creatine from guanidinoacetic acid in liver was originally demonstrated by Borsook & Dubnoff (1940*a*, *b*), and these authors later concluded that guanidinoacetic acid was not a normal constituent of that organ (Borsook & Dubnoff, 1941). This conclusion was confirmed by Bodansky, Duff & McKinney (1941), who reported that no guanidinoacetic acid could be detected in normal animals. However, Melville & Hummel (1951) reported 3·3–11·0 mg. of this acid/100 g. of rabbit liver, compared with a creatine content of 4–26 mg./100 g. These authors estimated guanidinoacetic acid by a modification (Macpherson, 1942) of the Sakaguchi reaction, after destruction of the arginine present with arginase. Eden, Harrison & Linnane (1954*a*, *b*), using a method similar to that of Melville & Hummel (1951), reported appreciable amounts of guanidinoacetic acid in normal rat liver. The last-mentioned findings were at variance with the early reports as well as with some observations made in this Laboratory. In view of this, and because of the considerable error which would be introduced into the estimation of liver creatine (Ennor & Stocken, 1948) if large amounts of guanidinoacetic acid were present, the problem has been reinvestigated.

The subject-matter of this paper was presented at a meeting of the Australian Biochemical Society in Adelaide, August, 1958.

EXPERIMENTAL

All guanidine compounds used were as previously described (Rosenberg, Ennor & Morrison, 1956). All organic solvents used in chromatography were purified by fractional distillation.

Preparation of tissue extracts. Rats, starved for 16 hr., were killed by cervical dislocation and the livers removed, rinsed in water and weighed. Single or pooled livers were dispersed in a Potter–Elvehjem type of homogenizer with 5 vol. of 1·5N-$HClO_4$. The suspension was centrifuged and the supernatant filtered. The residue was re-extracted with 3 vol. of 1·5N-$HClO_4$ and the supernatant, after filtration, combined with the original extract. The final volume of the pooled extracts did not exceed ten times that of the original tissue. A small sample (5–10 ml.) was removed and adjusted to pH 7·2 by the addition of 10N-KOH. The mixture was cooled to 0° and filtered to remove $KClO_4$. This neutralized extract was used for the colorimetric estimation of total guanidine.

The remainder of the extract was then passed through a column containing Zeo-Karb 225 ion-exchange resin, H^+ form. The volume of the resin bed was about 20 ml./250 ml. of extract, and with a flow rate of 3 ml./min. the effluent was free from guanidines. The column was then washed with water and eluted with aq. 2·5N-NH_3 soln. (flow rate, 1 ml./min.). The effluent was collected in 2 ml. fractions and the first guanidine-positive fraction appeared when the pH of the effluent rose above 7. All guanidine-positive fractions were pooled and the excess of ammonia was removed by vigorous boiling under reduced pressure. The solution was then adjusted to pH 6·8 with 5N-HCl and freeze-dried.

The dry residue was dissolved in the minimum volume of water (about 1 ml./20 g. of liver), cooled to 0° and a small amount of insoluble material removed by centrifuging. This was washed with 0·2 ml. of cold water, centrifuged and the supernatant combined with the first extract. The residue was discarded and the combined extract evaporated under reduced pressure to near dryness. Water was added so that the solution contained the equivalent of 10 g. of liver/0·2 ml. This concentrated extract was used for chromatography.

Apart from the extracts described above, 'fortified' extracts were also prepared when a known amount of guanidinoacetic acid was added to the liver before extraction. The amount added was 1 mg. of the acid/100 g. of liver, i.e. about one-third of the minimum amount of guanidinoacetic acid claimed to be present in liver (cf. Melville & Hummel, 1951; Eden *et al.* 1954*b*).

Chromatography. The solvent systems used were: phenol–water (4:1, v/v) in an SO_2 atmosphere (Makisumi, 1952); pyridine–3-methylbutan-1-ol–water (8:4:7, by vol.) (Thoai, Olomucki, Robin, Pradel & Roche, 1956); propan-1-ol–2 M-sodium acetate–acetic acid buffer, pH 4·0 (7:3, v/v) (Caldwell, 1955). All chromatograms were run in the ascending direction, on Whatman no. 3 MM papers (28 cm. × 28 cm.). Unless otherwise stated, 10 μl. of the concentrated extract (equivalent to 0·5 g. of tissue) or 10 μl. of a solution of reference compound (1 mg./ml.) was applied to each spot.

Estimation and detection of guanidines. Quantitative estimation of guanidines was carried out as described by Rosenberg *et al.* (1956) with aqueous reagent or the reagent containing 20% of propanol. For detection of compounds on paper the propanol-containing reagent (2 vol.) was mixed with 3 N-NaOH (1 vol.) and water (1 vol.) immediately before spraying. Pink spots on a white background were produced by all guanidines within 10 min. of spraying and maximum development took place in about 25 min. Specific detection of monosubstituted guanidines was achieved by the use of a modified Sakaguchi reagent as described by Acher & Crocker (1952). With this reagent it was necessary, with chromatograms run in phenol, to dry the papers overnight in a current of warm air, and then to wash in ether to reduce the dark background which otherwise resulted.

RESULTS

Eggleton, Elsden & Gough (1943) observed that the molecular extinction produced by creatine (and other asymmetrically disubstituted guanidines) in the Barrit reaction was approximately nine times that produced by monosubstituted guanidines. Rosenberg *et al.* (1956) described a modified reagent, containing α-naphthol, diacetyl and propanol, which increased the molecular extinctions of monosubstituted guanidines five- to nine-fold, without having any appreciable effect on the extinctions of the asymmetrically disubstituted guanidines. It was therefore thought that the ratio of extinctions obtained with any tissue extract in the presence and absence of propanol should provide some indication of the nature of the guanidines present.

Total guanidine was estimated by the two methods on normal rat-liver extracts, on such extracts to which guanidinoacetic acid had been added and on 'fortified' extracts. The extinctions given by the aqueous and propanol-containing reagents were compared and the ratio of the two extinctions was calculated for each type of extract. Similar comparisons were made with model systems, containing either creatine or guanidinoacetic acid, or a mixture of the two compounds. The results are presented in Table 1.

With creatine the ratio of the extinction in the presence of propanol to that in the aqueous reagent was slightly greater than unity. A similar ratio was found in the normal rat-liver extract. The corresponding ratio for guanidinoacetic acid was 5·3, and for a mixture of the two guanidines an intermediate value was obtained. A similar intermediate value was obtained when guanidinoacetic acid was added either to the normal extract or to the liver. There was thus no apparent loss of guanidinoacetic acid during the preparation of the liver extract. Similar results were obtained in six experiments.

Chromatography of liver extracts. Although the method described above established the absence of guanidinoacetic acid in liver in an amount comparable with that of creatine, it was not accurate enough to exclude the presence of small amounts of the compound. For the paper-chromatographic separation of the guanidines the phenol–water–SO_2 solvent gave excellent resolution of creatine, guanidinoacetic acid and arginine with R_g 0·75, 0·58 and 0·36 respectively. Extracts, prepared as described above, were chromatographed in this system. No guanidinoacetic acid was detected in

Table 1. *Extinction values of various compounds and mixtures in the Barrit reaction, with and without propan-1-ol*

	Extinction in aqueous reagent (*A*)	Extinction with 20% propan-1-ol (*B*)	*B*/*A*
Creatine (20 μg.)	0·250	0·290	1·16
Guanidinoacetic acid (20 μg.)	0·041	0·217	5·3
Creatine (20 μg.) + guanidinoacetic acid (20 μg.)	0·277	0·499	1·8
Normal liver extract (1 ml.)	0·060	0·069	1·15
Normal liver extract (1 ml.) + guanidinoacetic acid (20 μg.)	0·095	0·275	2·9
'Fortified' extract (1 ml.) (see text)	0·092	0·251	2·7

the normal extract, although excellent resolution was obtained when a guanidinoacetic acid marker was spotted together with the liver extract. A guanidinoacetic acid spot comparable in strength with that of creatine was also given by the fortified extract, indicating that, if present originally, this compound would not have been lost in the extraction procedure and subsequent treatment. In the chromatogram shown in Fig. 1 (*A*) the amount of extract applied was equivalent to 1·8 g. of liver. An extremely faint spot in the position of guanidinoacetic acid was then apparent. By visual comparison with the standard reference spot it was concluded that the amount in rat liver did not exceed 2 μg./g.

As the claims (Melville & Hummel, 1951; Eden *et al.* 1954*a*, *b*) for the presence of guanidinoacetic acid in liver were based on a positive Sakaguchi reaction after treatment with arginase, attempts were made to demonstrate chromatographically the presence of such compounds. The result is shown in Fig. 1 (*B*). Three faintly positive spots were apparent, but none corresponded with guanidinoacetic acid. The slowest spot (*c*) was identified as arginine and the fast-running one (*a*) had an R_F similar to that of γ-guanidinobutyric acid or methylguanidine. Spot (*b*) corresponded in position to β-guanidinopropionic acid. Since detection of the spots with the Sakaguchi reagent in the presence of phenol was not satisfactory, other solvents were employed. The propan-1-ol–sodium acetate buffer solvent afforded good separation of the three weak components from creatine, and the α-naphthol reagent could thus be used successfully for detection. In this solvent the slowest-moving component of the three again corresponded to arginine, the fastest component behaving as γ-guanidinobutyric acid, although the possibility of methylguanidine was not excluded. The intermediate component was identified by R_F and by reinforcement with a reference compound as β-guanidinopropionic acid (Fig. 2). To check the possibility of the fast component's being methylguanidine, the extract was chromatographed in pyridine–3-methylbutan-1-ol–water, where satisfactory separation of γ-guanidinobutyric acid (R_F 0·31) and methylguanidine (R_F 0·52) was obtained. An extremely faint spot in the position of methylguanidine was detected and was reinforced by the methylguanidine reference standard. A rough estimate, from spot size, suggested that the contribution of methylguanidine to the unknown fast spot [*a*, Fig. 1 (*B*)] was less than one-tenth.

DISCUSSION

The results leave no doubt that creatine is the only major substituted guanidine present in rat liver. Of the five additional guanidines which have been detected, three are present in readily detectable amounts: one has been identified as arginine and the other two, tentatively, as β-guanidinopropionic acid and γ-guanidinobutyric acid. The two remaining compounds were detected only with difficulty because of the extremely small amounts present, but from their chromatographic behaviour

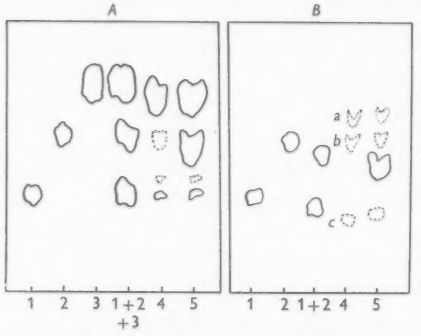


Fig. 1. Paper chromatography of liver extracts in phenol–water (4:1, v/v) in SO_2 atmosphere. The spots were developed with the diacetyl-α-naphthol reagent (*A*) or with the Sakaguchi reaction (*B*). 1, Arginine; 2, guanidinoacetic acid; 3, creatine; 4, liver extract; 5, 'fortified' liver extract. (Broken outlines denote faint spots.) Spots *a*, *b* and *c* are referred to in the text.

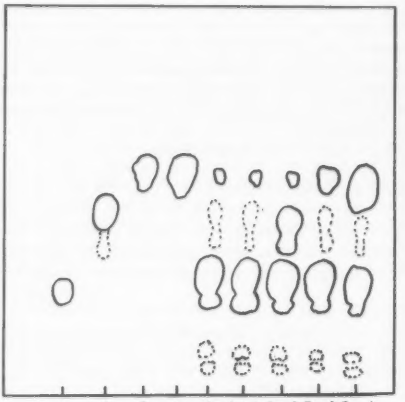


Fig. 2. Paper chromatography of liver extracts in propan-1-ol–2M-sodium acetate–acetic acid buffer, pH 4 (7:3, v/v). The spots were developed with the diacetyl-α-naphthol reagent. 1, Arginine; 2, β-guanidinopropionic acid; 3, γ-guanidinobutyric acid; 4, methylguanidine; 5, liver extract. (Broken outlines denote faint spots.)

there seems little doubt that they are methylguanidine and guanidinoacetic acid. Control experiments in which the acid was added either to the liver before extraction or to the extract after preparation indicate clearly that guanidinoacetic acid, if present in larger amounts than detected, would have been recovered after column treatment. It must be concluded therefore that guanidinoacetic acid is present in rat liver in only minute amounts, as indeed would be anticipated with an intermediate which is completely methylated in the presence of adequate amounts of methyl donor (cf. Cantoni & Vignos, 1954). The result, however, is in contrast with those obtained by Melville & Hummel (1951) and Eden *et al.* (1954*a*). The former authors based their conclusions on the detection of Sakaguchi-positive material in extracts treated with arginase to remove arginine. However, even if the arginine were not so removed, the amount of Sakaguchi-positive material present would not be sufficient to account for the result claimed and it is thus difficult to offer an explanation of their results. It is equally difficult to explain the results of Eden *et al.* (1954*a*), for, whereas the chromatographic solvent system which they used does not separate creatine and guanidinoacetic acid satisfactorily, the latter was detected by spraying with the Sakaguchi reagent, which does not react with creatine.

Although the evidence presented for the occurrence of γ-guanidinobutyric acid and β-guanidinopropionic acid is not conclusive, the presence of the former was to be expected in view of the results of Irreverre, Evans, Hayden & Silber (1957) and Pisano, Mitoma & Underfield (1957). The amounts present have been calculated to be about 3 μg./g., which is of the same order of magnitude claimed by Irreverre *et al.* (1957) for γ-guanidinobutyric acid in dog liver. In view of the small amounts of these guanidines (other than creatine and arginine) present in rat liver, it seems unlikely that a more positive and satisfactory identification will be achieved. Some experiments were carried out with large amounts of ox liver, but no guanidines other than creatine and arginine could be detected.

SUMMARY

1. The claim that guanidinoacetic acid is present in the liver in amounts comparable with those of creatine could not be confirmed. Chromatographic evidence indicated that the concentration of this compound in rat liver was below 2 μg./g.

2. The major substituted guanidine found in rat liver is creatine. Small amounts of arginine were also found. Two other compounds, tentatively identified as β-guanidinopropionic acid and γ-guanidinobutyric acid, were present in amounts not exceeding 3 μg./g. of tissue.

I wish to thank Professor A. H. Ennor for his advice in the course of this work and I am indebted to Dr J. F. Morrison of this Department for making available comprehensive charts on the chromatographic behaviour of various guanidines. Mr B. Thorpe provided valuable technical assistance.

REFERENCES

Acher, R. & Crocker, C. (1952). *Biochim. biophys. Acta*, **9**, 704.

Bodansky, M., Duff, V. B. & McKinney, M. G. (1941). *J. biol. Chem.* **140**, 365.

Borsook, H. & Dubnoff, J. W. (1940*a*). *J. biol. Chem.* **132**, 559.

Borsook, H. & Dubnoff, J. W. (1940*b*). *J. biol. Chem.* **134**, 635.

Borsook, H. & Dubnoff, J. W. (1941). *J. biol. Chem.* **138**, 389.

Caldwell, P. C. (1955). *Biochem. J.* **60**, xii.

Cantoni, G. L. & Vignos, jun., P. J. (1954). *J. biol. Chem.* **209**, 647.

Eden, E., Harrison, D. D. & Linnane, A. W. (1954*a*). *Aust. J. exp. Biol. med. Sci.* **32**, 333.

Eden, E., Harrison, D. D. & Linnane, A. W. (1954*b*). *Aust. J. exp. Biol. med. Sci.* **32**, 341.

Eggleton, P., Elsden, S. R. & Gough, N. (1943). *Biochem. J.* **37**, 526.

Ennor, A. H. & Stocken, L. A. (1948). *Biochem. J.* **42**, 557.

Irreverre, F., Evans, R. L., Hayden, A. R. & Silber, R. (1957). *Nature, Lond.*, **180**, 704.

Macpherson, H. T. (1942). *Biochem. J.* **36**, 59.

Makisumi, S. (1952). *J. chem. Soc. Japan*, **73**, 737. Quoted in *Chem. Abstr.* (1953), **47**, 5848.

Melville, R. S. & Hummel, J. P. (1951). *J. biol. Chem.* **191** 383.

Pisano, J. J., Mitoma, C. & Underfield, S. (1957). *Nature, Lond.*, **180**, 1125.

Rosenberg, H., Ennor, A. H. & Morrison, J. F. (1956). *Biochem. J.* **63**, 153.

Thoai, N. V., Olomucki, A., Robin, Y., Pradel, L. A. & Roche, J. (1956). *C.R. Soc. Biol., Paris*, **150**, 2160.

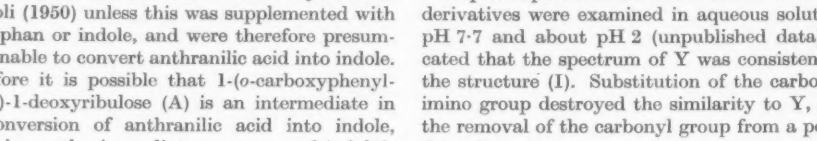
1-(*o*-Carboxyphenylamino)-1-deoxyribulose

A COMPOUND FORMED BY MUTANT STRAINS OF *AEROBACTER AEROGENES* AND *ESCHERICHIA COLI* BLOCKED IN THE BIOSYNTHESIS OF TRYPTOPHAN

By C. H. DOY and F. GIBSON
School of Bacteriology, University of Melbourne, Carlton, N. 3, Victoria, Australia

(*Received* 29 *July* 1958)

This paper presents the evidence for assigning the structure 1-(*o*-carboxyphenylamino)-1-deoxyribulose to a compound (Y) formed by mutant strains of *Aerobacter aerogenes* (N 3–33, N 2–18) and *Escherichia coli* (R 19–33). The experiments with *A. aerogenes* have been reported briefly (Gibson, Doy & Segall, 1958). None of these strains would grow in the minimal medium described by Davis & Mingioli (1950) unless this was supplemented with tryptophan or indole, and were therefore presumably unable to convert anthranilic acid into indole. Therefore it is possible that 1-(*o*-carboxyphenylamino)-1-deoxyribulose (A) is an intermediate in the conversion of anthranilic acid into indole, probably as the immediate precursor of indolylglycerol (Yanofsky, 1956), thus:



(A) (enol form)

RESULTS

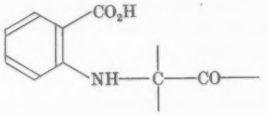
Formation and concentration of compound Y

The natural compound (Y) was readily formed in an aerated mixture of washed cells of *A. aerogenes* N 3–33 with glucose, ammonium chloride, magnesium sulphate and phosphate buffer. After centrifuging the cells compound Y was separated by acidification and extraction of the supernatant with ethyl acetate. Concentrates of the compound could then be prepared by evaporating to small bulk under vacuum, but complete removal of the solvent resulted in decomposition of the compound, as indicated by changes in the spectrum. It has not been found possible to isolate Y, other than as an impure oil, except on paper chromatograms; but its identity has been established by spot reactions on paper chromatograms, isolation of a crystalline 2:4-dinitrophenylhydrazone, ultraviolet-absorption spectroscopy and by a comparison with synthetic compounds.

Preliminary spectroscopic and chromatographic evidence for the structure of compound Y

The intense blue–violet fluorescence and the absorption spectrum suggested that the compound might be a derivative of anthranilic acid. Preliminary experiments in which the ultraviolet-absorption spectra of a number of anthranilic acid derivatives were examined in aqueous solution at pH 7·7 and about pH 2 (unpublished data) indicated that the spectrum of Y was consistent with the structure (I). Substitution of the carboxyl or imino group destroyed the similarity to Y, as did the removal of the carbonyl group from a position β to nitrogen.

The colour reactions of Y on paper chromatograms indicated that a sugar fragment was present and that this was probably a pentulose (Table 1). Concentrates of Y instantly decolorized alkaline methylene blue at room temperature, which suggested an Amadori compound (Hodge, 1955).



(I)

To detect Y on paper chromatograms it was more convenient to use alkaline 2:3:5-triphenyltetrazolium chloride, which gave an instantaneous red colour without heating. Under these conditions the common sugars, with the exception of fructose, produced only a faint coloration. Fructose, however, was easily differentiated from Y by the bright yellow it gave with anthrone reagent.

Comparison of the properties of glycosylamines and Amadori compounds with compound Y

As intermediates in the syntheses of Amadori compounds and as models for the study of absorption spectra, the glycosylamines, *N*-*o*-carboxyphenyl-D-ribosylamine, -D-arabinosylamine, -L-arabinosylamine, -D-xylosylamine and -D-glucosylamine, were prepared. These gave no colour with 2:3:5-triphenyltetrazolium chloride reagent. The different colour reactions (Table 1), susceptibility to acid hydrolysis and different ultraviolet-absorption spectra (Tables 3, 4; Figs. 1, 2) eliminated the possibility that Y was identical with one of these glycosylamines.

The glycosylamines when refluxed in *NN*-dimethylformamide rapidly yielded a reaction mixture giving the alkaline methylene blue and 2:3:5-triphenyltetrazolium chloride reactions typical of Y. Each reaction product gave a spot on paper chromatograms strongly fluorescent in ultraviolet light and an intense red with 2:3:5-triphenyltetrazolium chloride reagent at room temperature. When alternate spots of the unknown and reference compounds were chromatographed under identical conditions it was found that the substance originating from the ribosylamine, or the arabinosylamine, could not be distinguished from Y either by R_F (Table 2) or colour reactions (Table 1). The analogous compound from the xylosylamine always had a slightly lower R_F, but gave similar colour reactions, whereas that from the glucosylamine was readily differentiated by both R_F and colour reactions. These results were observed in the solvent systems methanol–butan-1-ol–benzene–water and butan-1-ol–acetic acid–water. A reference substance, usually Y, was included on all paper chromatograms and no reliance was placed on absolute R_F values alone since the latter varied slightly from day to day.

The ribosylamine (II) and the arabinosylamine (III) differ only in the configuration of C-2 in the sugar fragment, which difference is lost in the Amadori rearrangement to (V). However, the xylosylamine (IV) differs from both (II) and (III) at C-3 and this difference is retained in the Amadori compound (VI). This supports the relationship found on paper chromatograms. Although (II)–(VI) are drawn with the D configuration, none of the evidence so far presented indicates the configuration of Y. Further support for the Amadori structure was obtained from a detailed study of the ultraviolet-absorption spectra of natural and synthetic products and the preparation of a crystalline 2:4-dinitrophenylhydrazone from both natural and synthetic sources.

The ultraviolet-absorption spectra of the glycosylamines (Table 3, Fig. 1) showed characteristic

Table 1. *Colours developed with reagents sprayed on paper chromatograms*

Reagent	Compound: Pentoses	Compound Y and the synthetic pentose Amadori compounds	Synthetic hexose Amadori compound	*N*-*o*-Carboxyphenyl-D-pentosylamines*	*N*-*o*-Carboxyphenyl-D-glucosylamine*	Reference§
Ammonium molybdate	Blue	Blue	Blue	Blue	Blue	Aronoff & Vernon (1950)
Aniline acetate	Red† changing to red–brown	Red–brown	Pale yellow–brown	Red–brown	Pale yellow–brown	Aronoff (1956) Tracey (1950)
Phloroglucinol	Dark green (keto); purple (aldo)	Dark green	Yellow–brown	Pink, changing to purple	Yellow	Borenfreund & Dische (1957)
Anthrone	Bright purple (ketoxylose)	Grey–yellow, often changing to purple	Bright yellow	Brown	Brown	Johanson (1953)
Alkaline 2:3:5-triphenyltetrazolium chloride	Nil‡	Red	Red	Nil	Nil	Trevelyan, Procter & Harrison (1950)

* The glycosylamines were spotted on to Whatman no. 1 paper and not chromatographed.
† Used by the original authors as a quantitative colorimetric method in solution. When applied to paper, as suggested by Aronoff (1956), a red first develops but then changes to red–brown. Glucose gave only pale brown in the same time (6 min. at 100°).
‡ No immediate colour with pentoses unless heat is applied.
§ The references give results of original authors for pentoses. The other results were obtained during the present work.

changes with alteration in pH which were important in differentiating them from the synthetic Amadori compounds (Table 4, Fig. 2). On adjusting the pH of solutions of the glycosylamines in phosphate buffer (pH 7·7) to pH $\leqslant$2·0, by the addition of hydrochloric acid, the maximum in the 250 mμ region is rapidly quenched. Simultaneously the 315–320 mμ maximum undergoes a bathochromic shift to the 330 mμ region, but is then also quenched. The final spectrum has no clear maximum, but if the pH is then adjusted to $\geqslant$ 12 with 10 N-sodium hydroxide maxima appear at 240–242 and 310–312 mμ, which are identical with the maxima of anthranilic acid at this pH. If some of the original solution at pH 7·7 is adjusted directly to pH $\geqslant$ 12 there is no change in the wavelength of maximum absorption. This suggests that, at strongly acid pH, hydrolysis to anthranilic acid is very rapid. It is known from other experiments that hydrolysis proceeds even at slightly alkaline pH. The pentose and hexose compounds cannot be readily differentiated by spectral behaviour (Table 3).

Table 2. *R_F values of anthranilic acid, compound Y and synthetic Amadori compounds derived from certain* N*-glycosylamines of anthranilic acid*

R_F values below were obtained together on the one sheet of Whatman no. 1 paper developed with methanol–butan-1-ol–benzene–water (2:1:1:1, by vol.); solvent travel approx. 19 cm.

Compound	Parent glycosylamine	R_F
Anthranilic acid	—	0·93
Compound Y (four samples)	—	0·85–0·86
1-(*o*-Carboxyphenylamino)-1-deoxy-D-ribulose	*N*-*o*-Carboxyphenyl-D-arabinosylamine	0·86
	N-*o*-Carboxyphenyl-D-ribosylamine	0·85
1-(*o*-Carboxyphenylamino)-1-deoxy-L-ribulose	*N*-*o*-Carboxyphenyl-L-arabinosylamine	0·85
1-(*o*-Carboxyphenylamino)-1-deoxy-D-xylulose	*N*-*o*-Carboxyphenyl-D-xylosylamine	0·84*
1-(*o*-Carboxyphenylamino)-1-deoxy-D-fructose	*N*-*o*-Carboxyphenyl-D-glucosylamine	0·80

* The small difference in R_F between the xylulose and ribulose compounds was confirmed on many chromatograms (see the Experimental section), whereas compound Y and the ribulose derivatives could not be differentiated.

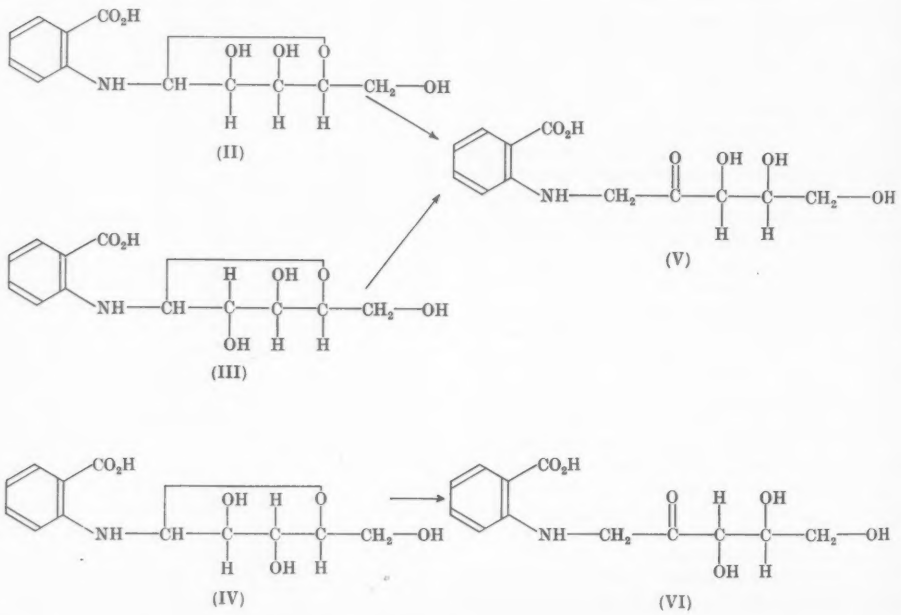


Table 3. *Ultraviolet-absorption spectra of anthranilic acid and some anthranilic acid glycosylamines*

Table 3. *Ultraviolet-absorption spectra of anthranilic acid and some anthranilic acid glycosylamines*

Spectra were measured on 0·1 mM-solutions against a blank of the appropriate solvent. Absorption was recorded at intervals of 5 mμ generally, 1 mμ at maxima, over the range 220–400 mμ.

	Solvent								
	0·1 M-Phosphate buffer, pH 7·7			Ethanol			Ethyl acetate–ethanol (98:2, v/v)		
Compound	$\lambda_{max.}$ (mμ)	$\epsilon_{max.}$	$\epsilon_{max.}$ ratio	$\lambda_{max.}$ (mμ)	$\epsilon_{max.}$	$\epsilon_{max.}$ ratio	$\lambda_{max.}$ (mμ)	$\epsilon_{max.}$	$\epsilon_{max.}$ ratio
N-*o*-Carboxyphenyl-D-ribosylamine	247 316	9500 3570	2·66/1	250 331	8930 4350	2·05/1	253 335	7750 4740	1·64/1
N-*o*-Carboxyphenyl-D-arabinosylamine	247 316	8950 3470	2·58/1	249–250 333	9750 4830	2·02/1	253 336	8300 5080	1·64/1
N-*o*-Carboxyphenyl-L-arabinosylamine	246 316	8850 3420	2·59/1	249–250 333	9980 4930	2·02/1	253–254 336	9050 5400	1·68/1
N-*o*-Carboxyphenyl-D-xylosylamine	247 316	9880 3650	2·7/1	249–250 333	9750 4830	2·02/1	253 336	7100 4250	1·67/1
N-*o*-Carboxyphenyl-D-glucosylamine	247 317	9600 3740	2·57/1	249 333	9500 4570	2·08/1	253 336	7570 4620	1·64/1
Anthranilic acid	240 310	7170 2980	2·41/1	248–249 336	7100 4460	1·59/1	252–253 336	5630 4900	1·15/1

Table 4. *Ultraviolet-absorption spectra of compound Y and synthetic Amadori compounds derived from certain N-glycosylamines of anthranilic acid*

Experimental samples were obtained as eluates from paper chromatograms. Eluates (eluting solvents 0·1 M-phosphate buffer, pH 7·7; ethanol; ethyl acetate) were diluted with pure solvent until an extinction value in the range 0·8–1·0 was recorded at 250 mμ. pH adjustments were made by adding conc. HCl or 10 N-NaOH to samples in phosphate at pH 7·7. Absorption was recorded at intervals of 5 mμ generally, 1 mμ at maxima, over the range 220–400 mμ.

		$\lambda_{max.}$ (mμ)					
		pH 7·7		Adjusted to			
Compound	Parent glycosylamine	Initial	Stored for approx. 16 hr., 20–25°	pH ⩽ 2*	pH ⩾ 12 (1 hr., 20–25°)	Ethanol	Ethyl acetate
1-(*o*-Carboxyphenylamino)-1-deoxy-D-ribulose	*N*-*o*-Carboxyphenyl-D-ribosylamine	250 324	240 309	250 343	240 308	254 347	254–255 346
	N-*o*-Carboxyphenyl-D-arabinosylamine	250 323	240 310	250 343	240 310	253–254 344–345	254 345
1-(*o*-Carboxyphenylamino)-1-deoxy-L-ribulose	*N*-*o*-Carboxyphenyl-L-arabinosylamine	250 325	240 310	250 345	242 308	254 347	254 346
1-(*o*-Carboxyphenylamino)-1-deoxy-D-xylulose	*N*-*o*-Carboxyphenyl-D-xylosylamine	249 325	240–242 312	249 345	242 310	254 347	254 343
1-(*o*-Carboxyphenylamino)-1-deoxy-D-fructose	*N*-*o*-Carboxyphenyl-D-glucosylamine	252 328	250 325	253 347–348	246–247 318	255 350	255 350
Y	—	250 325	240–242 310	250–251 345	240–242 310	254 346–347	254–255 345–347

* A weak inflexion is found at approx. 280 mμ.

In general the spectra of the synthetic Amadori compounds (Table 4, Fig. 2), as determined on eluates from paper chromatograms, bear the expected relationship to the spectra of the glycosylamines. The introduction of the additional chromophore has resulted in a bathochromic shift of both bands of maximum absorption, but more especially the band at longer wavelength, which has shifted 8–10 mμ (phosphate buffer, pH 7·7). In phosphate buffer (pH 7·7) Y has absorption maxima at 250 and 325 mμ. On adjusting the pH of this solution to $\leqslant 2$ with concentrated hydrochloric acid, the spectrum changes with the maxima now found at 250–251 and 345 mμ. An inflexion found at about 280 mμ (Fig. 2), but with weak absorption, is probably attributable to the free carbonyl group of the keto form. If some of the original solution at pH 7·7 is adjusted to pH $\geqslant 12$, with 10 N-sodium hydroxide, immediate spectral changes occur with a gradual shift (over about 60 min.) to a maximum at 310–312 mμ and an inflexion or slight maximum at 240–242 mμ. Readjustment to slightly acid pH results in a spectrum with no prominent features.

Paper chromatography of the Amadori reaction products from each of the pentosylamines separated compounds (2:3:5-triphenyltetrazolium chloride positive) which on elution had identical spectra with natural Y, isolated and eluted in the same manner (Table 4, Fig. 2). The hexose analogue derived from the glucosylamine exhibited similar spectra when examined at slightly alkaline and strongly acid pH. However, the behaviour at strongly alkaline pH was distinctive (Table 4). The spectral changes were similar to, but slower than, those observed with the pentose compounds. This differentiation was more distinct at pH 7·7 as the rate of change was retarded still further. After elution with buffer, pH 7·7, the spectra measured immediately were similar, but after 16 hr. at room temperature, fresh examination indicated that the samples of Y and the synthetic pentose Amadori compounds now exhibited a spectrum with a maximum at 310–312 mμ and an inflexion, or slight maximum, at 240–242 mμ. In contrast, the synthetic hexose Amadori compound had shown little change, with maxima found at 250 and 325 mμ (Table 4, Fig. 2). These changes are interpreted as indicating the liberation of anthranilic acid after enolization of the Amadori compound. From the above observations it would seem that the fructose derivative is more stable to alkali than the pentose compounds. In general, this alkaline lability would be expected of Amadori compounds. The behaviour of Y was in all respects identical with that of the synthetic pentose Amadori compounds.

Preparation and properties of 2:4-dinitrophenylhydrazones from natural and synthetic products

As Y seemed unstable when attempts were made to purify the crude accumulation product, it was necessary to attempt the preparation and isolation of a derivative under mild non-alkaline conditions. Compound Y was quantitatively extracted from ethyl acetate with phosphate buffer (pH 7·7), and the addition of an acid solution of 2:4-dinitrophenylhydrazine to this extract resulted in the formation of an amorphous, orange–red precipitate, which on isolation melted over a wide range (100–200°). Recrystallization from ethanol eventually yielded rather amorphous yellow crystals, m.p. 213–215° (decomp.). Elementary analysis indicated a mono-2:4-dinitrophenylhydrazone,

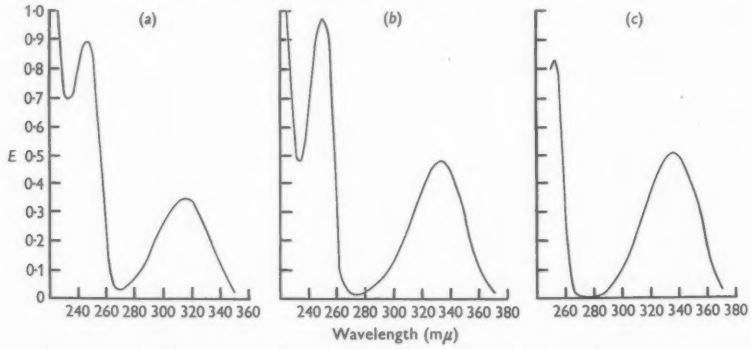


Fig. 1. Absorption spectra of 0·1 mM-*N*-*o*-carboxyphenyl-D-arabinosylamine measured in (*a*) 0·1 M-phosphate buffer, pH 7·7, max. 247, 316 mμ; (*b*) ethanol, max. 249·5, 333 mμ; (*c*) ethyl acetate–ethanol (98:2, v/v) max. 253, 336 mμ. These spectra are typical of the anthranilic acid glycosylamines prepared (see the Experimental section). For details of $\lambda_{max.}$, ϵ values and ratios see Table 3.

$C_{18}H_{19}O_9N_5$. An apparently identical compound was obtained by the addition of 2:4-dinitrophenylhydrazine reagent to the Amadori-rearrangement reaction mixture obtained from *N*-*o*-carboxyphenyl-D-arabinosylamine. The synthetic and naturally derived hydrazones gave no depression of melting point on admixture, could not be separated by paper chromatography and showed identical ultraviolet-absorption maxima (Fig. 3). At pH $\leqslant$ 2 the spectral changes immediately and on storage were identical.

Although neither the natural nor the synthetic compound has been isolated, the method of preparation of the synthetic compound, the typical

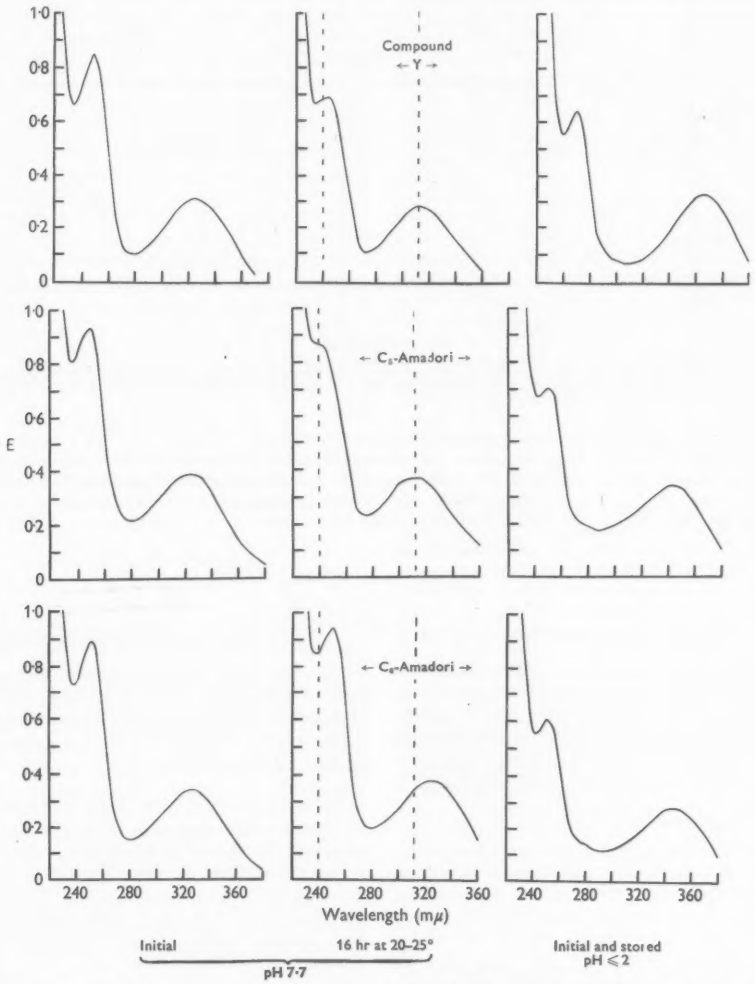


Fig. 2. Comparison of the absorption spectra of compound Y and the synthetic Amadori compounds, showing the identical behaviour of Y and the Amadori compounds derived from *N*-*o*-carboxyphenyl-pentosylamines. The hexose analogue, derived from *N*-*o*-carboxyphenyl-D-glucosylamine, is differentiated by the rate of hydrolysis at pH 7·7, which is slower for the hexose compound. Experimental samples were eluted from paper chromatograms with 0·1 M-phosphate buffer, pH 7·7. For details of $\lambda_{max.}$ see Table 4, and for preparation of samples see the Experimental section.

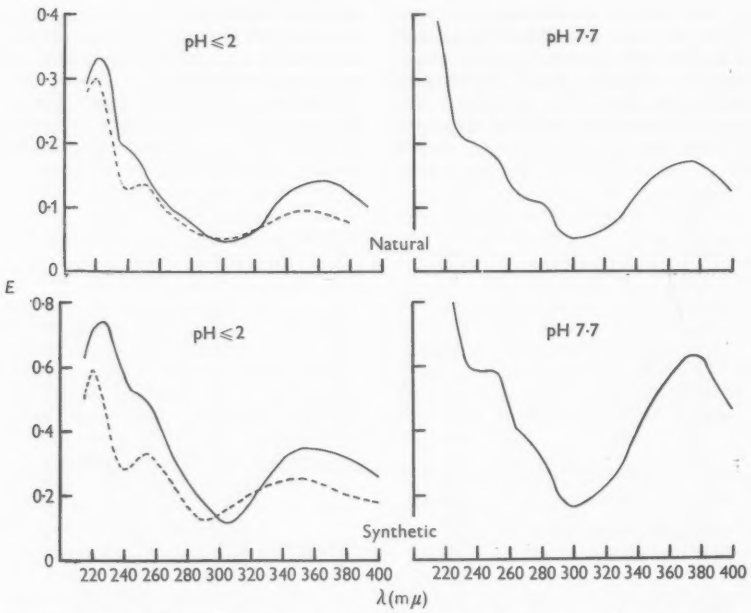
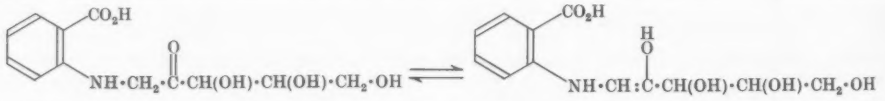


Fig. 3. Absorption spectra of the 2:4-dinitrophenylhydrazone derivatives from compound Y and a synthetic source of 1-(*o*-carboxyphenylamino)-1-deoxy-D-ribulose. The spectrum at pH ≤ 2 was recorded after adjusting the pH of a solution in 0·1 M-phosphate buffer, pH 7·7, with conc. HCl, and initially shows rapid changes. ——, Spectrum recorded immediately; - - - -, spectrum after 2 days' (natural), overnight (synthetic) storage at pH ≤ 2. $\lambda_{max.}$ (pH 7·7): 245, 280, 373 mμ. Spectra are qualitative (different *E* values).



reaction with redox reagents and the behaviour of products from the pentosylamines, together with the evidence of the 2:4-dinitrophenylhydrazones, support the view that Y is an Amadori compound with the structure 1-(*o*-carboxyphenylamino)-1-deoxyribulose (1-anthranilino-1-deoxyribulose).

Configuration of compound Y

Attempts were made to assign a configuration to the molecule. Paper chromatography did not differentiate the D and L synthetic compounds, although this was attempted with optically active substances either impregnated on the paper or present in the developing solvent. Attempts to obtain the specific rotation of the 2:4-dinitrophenylhydrazones failed because of the intense colour in pyridine. A study of the optical rotations of the glycosylamines in pyridine showed that the D-pentosylamines were laevorotatory and suggested that the D-Amadori compound would behave similarly. The optical rotation of an ethyl acetate concentrate of Y was *laevo*. This indirect evidence is not considered sufficient to assign the D configuration definitely to 1-(*o*-carboxyphenylamino)-1-deoxyribulose.

Formation of compound Y by Escherichia coli

Towards the conclusion of this work a mutant strain (R 19–33) of *E. coli* was found to accumulate a compound shown by ultraviolet-absorption spectroscopy, colour reactions and R_F values to be identical with the 1-(*o*-carboxyphenylamino)-1-deoxyribulose accumulated by the *A. aerogenes* strains.

DISCUSSION

Yanofsky (1956) has postulated 1-(*o*-carboxyphenylamino)-1-deoxyribulose as an intermediate in tryptophan biosynthesis, suggesting that the phosphorylated compound is involved in the con-

version of anthranilic acid into indolyl 3-glycerol phosphate. At the same time he suggested *N*-*o*-carboxyphenylribosylamine 5-phosphate as the immediate precursor of 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate. We have found no evidence to suggest that the compound as accumulated was phosphorylated. It is possible that it is phosphorylated within the cells, since Lingens, Burkhardt & Hellmann (1957) have shown that indolylglycerol phosphate within the cells is excreted as indolylglycerol. Yanofsky (1956) has shown the formation of indolylglycerol phosphate from anthranilic acid by cell-free extracts of *E. coli*. That the ribosylamine was accumulated by N 3–33 but then underwent spontaneous conversion into the Amadori compound is unlikely. It has been shown that under the conditions of accumulation the ribosylamine readily hydrolyses to a diazotizable amine which has an ultraviolet spectrum identical with that of anthranilic acid.

Intermediates in biosynthetic pathways have usually been isolated after the accumulation in culture fluids. The results above would suggest that the use of cell suspensions may be of particular value in the detection and isolation of more labile compounds.

EXPERIMENTAL

All melting points are corrected. Elementary analyses are by The Australian Microanalytical Service, Commonwealth Scientific and Industrial Research Organization, Division of Industrial Chemistry, Melbourne.

Accumulation of compound Y

A light suspension (approx. 4×10^9 cells/ml.) of *A. aerogenes* N 3–33 was made in water, from the growth on a heart-infusion–agar slope (Gibson, Jones & Teltscher, 1956) incubated for 16 hr. at 37°. Thirty heart-infusion–agar plates (4 in. diam.) were inoculated by spreading 0·2 ml. of the suspension over the surface of each plate. The inoculated plates were incubated for 16 hr. at 37° and the cells washed off with a total of 5 ml. of 0·9% NaCl soln./plate. Freshly harvested cells were used for each experiment. The cells were centrifuged and washed twice with 0·9% NaCl soln., resuspended in a mixture of substrates (Na_2HPO_4, 25·6 g.; KH_2PO_4, 2·72 g.; D-glucose, 36 g.; 0·1 M-$MgSO_4$, 2 ml.; water to a total volume of 2 l.); the turbidity was measured with an EEL portable colorimeter (625 mμ filter, galvanometer-scale reading 17–22, with ½ in. diam. tubes). The mixture was incubated for 24 hr. at 37° with vigorous aeration through a sintered-glass disk. Cells were then removed by centrifuging the accumulation mixture in a Sharples supercentrifuge to give a clear supernatant.

Attempted isolation of compound Y

Extraction. The supernatant (approx. 1·8 l.) was acidified with conc. HCl (18 ml.) and was extracted by shaking with an equal volume of ethyl acetate. The aqueous layer was discarded and the strongly fluorescent organic layer dried over anhydrous Na_2SO_4 (overnight). After filtration the ethyl acetate extract was concentrated about tenfold by distillation of the solvent at about 60 mm. Hg. Such concentrates were somewhat yellow in colour and were the stock form in which Y was stored in the refrigerator.

Further procedure. Most of the remaining ethyl acetate was removed by continuing the distillation at reduced pressure. Usually a rather waxy material would separate during concentration, especially if the concentrate was chilled. Any such precipitate was filtered off and discarded. As concentration proceeded the colour deepened until finally a dark-red oil was obtained. This residue would not crystallize, even after prolonged storage in the 'deep-freeze', but degenerated to a dark semi-solid mass. The original oil rapidly decolorized methylene blue and gave an intense red with 2:3:5-triphenyltetrazolium chloride at room temperature (20–25°). It produced an oily red precipitate with 2:4-dinitrophenylhydrazine reagent (0·5% in 2 N-HCl).

Alternatively the ethyl acetate concentrate was extracted with an equal volume of aqueous 0·1 M-phosphate buffer (Na_2HPO_4–KH_2PO_4; pH 7·7) to remove Y quantitatively. Compound Y could be concentrated by reducing the quantity of buffer used in the extraction. The buffer extract was acidified (0·1 ml. of conc. HCl/10 ml. of buffer) and extracted with an equal volume of ethyl acetate. If the organic solvent was removed at reduced pressure the product was a red oil with similar properties to the oil isolated from the primary ethyl acetate extract. It was found that Y could best be precipitated as its 2:4-dinitrophenylhydrazone by the addition of 2:4-dinitrophenylhydrazine reagent to the buffer extracts (see below).

Paper chromatography

Paper chromatograms (Whatman no. 1 or no. 3 paper) were developed with ascending solvent. Because of slight day-to-day variation in the R_F of a given compound it was thought advisable to rely on comparison of R_F values with a reference compound on each sheet. The reference compound was usually Y, applied as the stock ethyl acetate concentrate; however, anthranilic acid and the common sugars were sometimes used, depending on the reason for running the chromatogram. For the chromatography of the 2:4-dinitrophenylhydrazones buffered paper was used to prevent 'tailing'. The paper was prepared by drawing the sheet through 0·1 M-phosphate buffer (pH 7·7), blotting to absorb the surplus and then allowing to dry at room temperature until slightly moist.

Solvent systems. The principal system used was methanol–butan-1-ol–benzene–water (2:1:1:1, by vol.; Mason & Berg, 1951). If two compounds appeared to have an identical R_F this was confirmed with the organic layer from butan-1-ol–acetic acid–water (5:4:1, by vol.). Butan-1-ol–propan-1-ol–water (1:2:1, by vol.) made 0·005 N with respect to ammonia (Partridge, Bonner & Yanofsky, 1952), and also butan-1-ol saturated with water, were used as solvents in some experiments. For development of 2:4-dinitrophenylhydrazones aqueous 83% ethanol was used (Kun & Garcia-Hernandez, 1957).

Detection of compounds. All the compounds of interest fluoresced in ultraviolet light and this property was used for their detection. In seeking a guide to chemical structure, colour reactions were carried out on the paper and the colour reactions of Y were compared with synthetic compounds (Table 1). Reagents were prepared as described by

the original authors but when heating was required to develop the colour it was more convenient to use an oven at 90–100° rather than to vary the temperature from test to test. These deviations in technique appeared only to alter the time required for development. Use was made of the strong reducing power of Amadori compounds to detect them on paper. The alkaline 2:3:5-triphenyltetrazolium chloride reagent of Trevelyan, Procter & Harrison (1950) was used. An intense red was instantaneously produced at room temperature. The common reducing sugars gave a similar colour but the development at room temperature was slow. It was found that the colour reactions were centred on the Y or synthetic Amadori compound spot detected in ultraviolet light and that sensitivity, as measured by area, was less than that observed with ultraviolet light. The fluorescence of the Amadori compounds was so intense that as chromatograms were developed it was often possible to follow migration in daylight.

Comparison of known compounds with compound Y. For this purpose pairs of Y spots were alternated with pairs of known compound along the origin. After development, vertical strips were cut so as to include a Y and known spot. A comparison of R_F was then made under ultraviolet light and a colour reaction carried out. Because spots were compared under identical conditions the results were comparable irrespective of minor variations in technique. All the colour reactions were reproducible, with the exception of the test with anthrone reagent, which sometimes gave a grey–yellow reaction and at other times a purple with Y and the synthetic pentose Amadori compounds. The hexose Amadori compound always gave a bright yellow with this reagent. The most characteristic apart from the 2:3:5-triphenyltetrazolium chloride reaction was that with the phloroglucinol reagent of Borenfreund & Dische (1957). For colour reactions see Table 1.

R_F values. It was impossible to differentiate 1-(*o*-carboxyphenylamino)-1-deoxy-D-ribulose from the L-compound by R_F or colour reaction. Although 1-(*o*-carboxyphenylamino)-1-deoxy-D-xylulose has only a slightly lower R_F than the ribulose analogue (Table 2) the two were readily differentiated. The difference was clearly seen when a series of sheets were chromatographed with alternate spot pairs of the two compounds.

Performance. In general, development with the solvents listed above gave well-defined spots; some tailing could be observed under ultraviolet light but was not usually detected with chemical reagents. The glycosylamines were difficult to chromatograph because of the tendency to hydrolyse to anthranilic acid and sugar. It cannot be said with certainty that a spot attributable to a glycosylamine was observed in this work.

Syntheses

Glycosylamines of anthranilic acid. In general, the preparations of the glycosylamines were based on the methods of Berger & Lee (1946). No difficulty was found in obtaining good yields under moderate conditions and without the addition of catalysts. Since maximum yields were not the primary concern, preparations were not exhaustively treated. It was found difficult to recrystallize the products and thorough washing with water and ethanol gave crystals at least the equal of recrystallized material (as indicated by melting point and elementary analysis). At all stages care was taken to exclude foreign matter.

N-*o*-*Carboxyphenyl*-D-*arabinosylamine.* Anthranilic acid (5·9 g.) in absolute ethanol (50 ml.) was added to D-arabinose (5·0 g.) and refluxed on a water bath. The sugar rapidly dissolved and after 5 min. the reaction mixture solidified. After cooling, the crystalline mass was filtered off, washed with ethanol (approx. 20 ml.), water (approx. 20 ml.) and a further few millilitres of ethanol. Drying over silica gel at room temperature and under vacuum gave a mass of fine white needles (9 g., 100% yield on D-arabinose), m.p. 167–168° (decomp.); $[\alpha]_D^{23}$ −62° in pyridine (*c*, 1·25, rotation recorded within 15 min. of solution), −48° (48 hr.) (Found: C, 53·7; H, 5·8; O, 36·0; N, 5·1. $C_{12}H_{15}O_6N$ requires C, 53·5; H, 5·6; O, 35·7; N, 5·2%).

N-*o*-*Carboxyphenyl*-L-*arabinosylamine.* This compound was prepared in the same manner as the D-arabinosylamine, but also as follows. L-Arabinose (0·2 g.) in water (1 ml.) was added to anthranilic acid (0·22 g.) in ethanol (2 ml.) and the mixture shaken at room temperature (22°). After 25 min. crystals began to separate, giving a semi-solid mass at 30 min. After removal from the shaker at 45 min., the product was left at room temperature overnight. After washing with ethanol (15 ml.), water (15 ml.) and a further few millilitres of ethanol the product was sucked dry and drying completed at room temperature over silica gel under vacuum. This treatment yielded fine white needles (0·31 g., 86·4% on L-arabinose), m.p. 161° (decomp.); $[\alpha]_D^{24}$ +61·2° in pyridine (*c*, 1·25, rotation recorded within 15 min. of solution), +52·3° (24 hr.), +48·5° (69 hr.) (Found: C, 53·4; H, 5·8; O, 35·8; N, 5·13. $C_{12}H_{15}O_6N$ requires C, 53·5; H, 5·6; O, 35·7; N, 5·2%).

N-*o*-*Carboxyphenyl*-D-*ribosylamine.* Anthranilic acid (0·2 g.) was stirred into a solution of D-ribose (0·2 g.) in 95% ethanol (2·7 ml.). The anthranilic acid rapidly dissolved and the mixture was allowed to stand at room temperature (20°) for 3 hr. and then overnight at 4°. During the next day the mixture became semi-solid but was kept at 4° for a further 3 days. The product was filtered off, washed with ethanol and then water, and sucked dry and finally dried at room temperature over silica gel under vacuum to give a mass of white needles (0·3 g., 81% on D-ribose), m.p. 121° (decomp.); $[\alpha]_D^{23}$ −58·2° in pyridine (*c*, 1·25; rotation recorded within 15 min. of solution), +2·6° (48 hr.) (Found: C, 52·2; H, 5·9; O, 37·4; N, 5·0. $C_{12}H_{15}O_6N,\frac{1}{2}H_2O$ requires C, 51·8; H, 5·8; O, 37·4; N, 5·0%).

N-*o*-*Carboxyphenyl*-D-*xylosylamine.* D-Xylose (0·21 g.) in 95% ethanol (1 ml.) was added to anthranilic acid (0·22 g.) in ethanol (2 ml.) and the mixture shaken for 15 min. at room temperature, by which time a mass of white crystals had separated. After standing overnight at room temperature the crystals were filtered off, washed with ethanol (30 ml.), water (30 ml.) and a further few millilitres of ethanol, and sucked dry and then dried at room temperature over silica gel under vacuum to give white needles (0·28 g., 78% on D-xylose), m.p. 167° (decomp.); $[\alpha]_D^{24}$ −42·6° in pyridine (*c*, 1·25, rotation recorded within 15 min. of solution), −23·4 (48 hr.) (Found: C, 53·2; H, 5·6; O, 35·5; N, 5·2. $C_{12}H_{15}O_6N$ requires C, 53·5; H, 5·6; O, 35·7; N, 5·2%).

N-*o*-*Carboxyphenyl*-D-*glucosylamine.* D-Glucose (5 g.) was added to anthranilic acid (5·7 g.) in ethanol (50 ml.) and the mixture refluxed on a water bath until slightly red in colour (15 min.). Some of the sugar was undissolved, but the bulk of the product was decanted and stored in the

refrigerator. Over a period of about 3 weeks the mixture became plastic and then partially solidified. The mass was filtered off and triturated with water, ethanol, water (each 100 ml.) and finally washed with a little ethanol. After suction the product was dried at room temperature over silica gel under vacuum to give white crystals (3·4 g., 39% on D-glucose), m.p. 137–138° (decomp.); $[\alpha]_D^{25}$ +102·6° in pyridine (*c*, 1·25, rotation recorded within 15 min. of solution), +81·9 (48 hr.) (Found: C, 49·2; H, 6·1; O, 40·5; N, 4·6. $C_{13}H_{17}O_7N,H_2O$ requires C, 49·2; H, 6·0; O, 40·4; N, 4·4%).

Rearrangement of the glycosylamines to Amadori compounds. This was accomplished by refluxing the glycosylamines in *NN*-dimethylformamide.

Glycosylamine (0·5 g.) in *NN*-dimethylformamide (2 ml.) was refluxed for about 5 min. During this time the mixture passed from colourless through yellow to deep red, at which stage the reaction mixture was cooled and poured into a test tube. Any remaining glycosylamine was decomposed by adding a drop of conc. HCl to the product and allowing the mixture to stand for several hours at room temperature. These products were used directly for paper-chromatographic separation of the Amadori compound. Chromatography indicated that the reaction products were anthranilic acid, the Amadori compound, the free sugar and at least two coloured compounds, one travelling at the front (methanol–butan-1-ol–benzene–water) and the other between anthranilic acid and the Amadori compound.

Properties of the glycosylamines. Solutions of the glycosylamines in pyridine, or *NN*-dimethylformamide, did not decolorize alkaline methylene blue or produce a colour with alkaline 2:3:5-triphenyltetrazolium chloride at room temperature. All the compounds showed a slow mutarotation in pyridine (not specially dried), but this was not followed to the equilibrium value. The specific rotation of *N*-*o*-carboxyphenyl-D-glucosylamine, H_2O was found to be of similar order but of opposite sign to that reported by Sannié (1948) in pyridine. Our material melted at 137–138° and that of Sannié at 125–127°. Inoue, Onodera & Kitaoka (1951–52) report a compound with m.p. 137–138°, $[\alpha]_D$ +68 in ethanol–water. The ultraviolet-absorption spectra (Table 3, Fig. 1) of the unrecrystallized but reasonably pure glycosylamines (see elementary analyses) showed no significant difference between the pentose and hexose compounds. Hydrolysis to anthranilic acid appeared to proceed rapidly at strongly acid pH. An experiment to determine the stability of the ribosylamine (5 mM in 0·067 M-phosphate buffer) at pH 8·04, 6·98, 5·91 and 5·29 at 37° indicated rapid hydrolysis at the lowest pH and about 50% hydrolysis, after 2¾ hr., at pH 8·04. The rate of hydrolysis increased with decrease in pH. Hydrolysis was followed by observing the hypsochromic shifts of the bands of maximum absorption (247 → 240 mμ, 316 → 310 mμ; 1 in 40 dilution measured in 0·1 M-phosphate buffer, pH 7·7) and the measurement of diazotizable amine by the method of Bratton & Marshall (1939). On the basis of a standard curve for anthranilic acid, the colour values indicated complete hydrolysis within 20 hr. at all pH values. The experiment indicated that if *N*-*o*-carboxyphenyl-D-ribosylamine was accumulated in substrates at approx. pH 7 it would finally be detected as anthranilic acid.

Properties of the Amadori compounds. The Amadori compounds were easily detected on paper chromatograms by the intense blue-violet fluorescence in ultraviolet light and the immediate formation of intense red when sprayed with 2:3:5-triphenyltetrazolium chloride (0·5% in $CHCl_3$) followed by ethanolic NaOH soln. Heating was unnecessary. Reaction products from the rearrangement of the glycosylamines rapidly decolorized alkaline methylene blue at room temperature (20–25°), and this property was evident in increasing degree from the time the rearrangement mixture refluxed. No extensive attempt was made to isolate the pure Amadori compounds other than on paper chromatograms. A dark-red oil was precipitated from rearrangements in pyridine or pyridine–ethyl malonate by addition of light petroleum (b.p. 40–60°). Such oils were very similar in behaviour to the oils obtained by purifying the natural product. None of these oils could be induced to crystallize. A 2:4-dinitrophenylhydrazone was prepared (see below) but was difficult to purify. Details of the ultraviolet-absorption spectra of the Amadori compounds is discussed elsewhere in this paper. The data obtained in a number of solvents and pH values are presented in Table 4 and Fig. 2. In contrast with the glycosylamines, the pentose and hexose compounds could be differentiated by their spectral behaviour and were less stable at alkaline pH than at acid. It was concluded that in alkali enolization occurred, followed by hydrolytic cleavage of the molecule. There was evidence from the spectral changes that anthranilic acid was one product of cleavage. From paper chromatograms the eluting power of solvents was in the descending order: 0·1 M-phosphate buffer (pH 7·7), ethanol, ethyl acetate.

Preparation of 2:4-dinitrophenylhydrazones

From naturally accumulated compound Y. The Y spot on paper chromatograms reacted fairly slowly with 2:4-dinitrophenylhydrazine reagent to give a deep orange and suggested the possibility of isolating a hydrazone from a bulk source. If reagent (0·5% 2:4-dinitrophenylhydrazine in 2N-HCl) was added to a solvent-free Y concentrate a viscous red oil and some amorphous orange crystals separated. Efforts to recrystallize this product failed. As described above, Y was easily obtained as a solution in 0·1 M-phosphate buffer (pH 7·7), and this was found to be the best medium from which to precipitate the 2:4-dinitrophenylhydrazone. An equal volume of 0·5% 2:4-dinitrophenylhydrazine in 2N-HCl was added to the buffer extract. The mixture rapidly became turbid and after about 1 hr. an amorphous, orange–red ppt. began to separate. After standing at room temperature overnight, the product was collected and dried (1–3 g.; m.p. 100–200°). Recrystallization of the crude product was difficult, probably because of interaction with the recrystallizing solvent. It was possible to recrystallize from ethanol with large losses of material, but subsequent concentration of the mother liquor yielded amorphous yellow crystals. These were washed with ethanol, sucked dry and finally dried over silica gel under vacuum (m.p. 213–215° decomp.). An elementary analysis was consistent with the product (VII*a*) being the mono-2:4-dinitrophenylhydrazone of a 1-(*o*-carboxyphenylamino)-1-deoxypentulose (Found: C, 48·0; H, 4·4; O, 32·0; N, 15·2. $C_{18}H_{19}O_9N_5$ requires C, 48·1; H, 4·2; O, 32·0 N, 15·6%).

From a synthetic source of 1-(*o-carboxyphenylamino*)-1-*deoxy*-D-*ribulose.* An Amadori-rearrangement reaction (see above) was carried out on *N*-*o*-carboxyphenyl-D-arabinosylamine and excess of 2:4-dinitrophenylhydrazone reagent

(0·5% 2:4-dinitrophenylhydrazine in 2N-HCl) was added to the products. The amorphous orange–red solid formed was washed with acetone and recrystallized from acetone to give yellow–orange needles 'blushing' to orange–red, m.p. 210–211° (decomp.) (Found: C, 47·9; H, 4·5; O, 32·2; N, 14·6%). Ethanol was added till nearly all the crystals were dissolved at the boiling point and the mixture was filtered whilst hot. The clear filtrate gave a few orange–yellow crystals (m.p. 211°, decomp.) after 2 days in the deep freeze. Addition of a little water to the mother liquor caused long needle-like crystals to form in association with some amorphous material. This material, after drying over silica gel under vacuum, had m.p. 214° (decomp.) (VII*b*) (Found: C, 47·6; H, 4·5; O, 32·5; N, 14·9. $C_{18}H_{19}O_9N_5$ requires C, 48·1; H, 4·2; O, 32·0; N, 15·6%). VII*a* ('natural') and VII*b* ('synthetic') gave no observable depression in mixed melting point although the analysis of the 'synthetic' material did not fit the expected structure as well as that of the 'natural' product. The ultraviolet-absorption spectra of (VII*a*) and (VII*b*) were very similar in phosphate buffer (pH 7·7). On adjustment to pH < 2 with conc. HCl the immediate and long-term changes were identical (Fig. 3). Paper chromatograms of (VII*a*) and (VII*b*) developed with aqueous 83% ethanol gave spots of identical R_F (0·58; solvent front travelled 19·4–20·4 cm.).

Ultraviolet-absorption spectroscopy

Spectra were obtained with a Unicam model SP. 500 spectrophotometer and 1 cm. silica cells. Solvents were commercial absolute ethanol, ethyl acetate (analytical reagent) and 0·1 M-phosphate buffer (pH 7·7) prepared from Na_2HPO_4 and KH_2PO_4 of analytical grade. Experimental samples were read against solvent from the same source as used to prepare the sample. Extinction values were recorded at 5 mμ intervals, except at maxima and some minima, when the interval was usually 2 mμ, but sometimes 1 mμ. Because the pH of experimental samples was often varied just before recording the spectrum, it was standard practice to record from short to long wavelengths. When available as crystals the experimental samples were weighed, made up and diluted in solvent to a concentration of 0·1 mM. With ethyl acetate it was necessary to make the primary solution in ethanol and then to add a portion to ethyl acetate to give a final concentration of 0·1 mM in ethyl acetate–ethanol (98:2, v/v). A solvent blank with these proportions was used. With the Amadori compounds crystals were not available and all spectra were determined on eluates from paper chromatograms. Eluates were adjusted to give an extinction of approx. 0·9 at 250 mμ. With ethyl acetate eluates the extinction was often below this value even without dilution, in which case the values could not be adjusted. After reading spectra in buffer at pH 7·7 it was usual to adjust the pH to ≤2 by adding a drop of conc. HCl and then to record the spectrum again. Similarly, adjustments were made, to pH ≥12, with 10N-NaOH, a fresh portion of the sample at pH 7·7 being used. The dilution effected by these additions (< 1%) was ignored. pH measurements were made with a Jones model B pH electrometer. It was particularly noticeable with the 2:4-dinitrophenylhydrazone derivatives that after acidification the spectrum was influenced by the time taken and the direction of recording. However, the behaviour was characteristic in itself and seen with derivatives from both natural and synthetic sources.

SUMMARY

1. A new compound was found to be accumulated by washed cell suspensions of mutant strains of *Aerobacter aerogenes* (N 3–33, N 2–18) and *Escherichia coli* (R 19–33) requiring tryptophan or indole for growth. This compound has been identified as 1-(*o*-carboxyphenylamino)-1-deoxyribulose and may be the immediate precursor of indolylglycerol phosphate in tryptophan synthesis.

2. Colour reactions of the natural compound (Y), isolated on paper chromatograms, indicated the presence of a sugar fragment, probably a pentulose. Reducing properties suggested an Amadori compound.

3. A number of glycosylamines of anthranilic acid were synthesized and used as model compounds for ultraviolet-absorption spectrophotometry and as parent compounds in Amadori-rearrangement reactions.

4. The spectral and other characteristics of the Amadori-rearrangement products were determined with material from paper chromatograms.

5. A comparison of the properties of the synthetic Amadori compounds with those of compound Y indicated that the latter was identical with 1-(*o*-carboxyphenylamino)-1-deoxyribulose. The differentiation from 1-(*o*-carboxyphenylamino)-1-deoxy-D-fructose was on the basis of colour reaction, R_F and stability at alkaline pH. A xylulose structure was rejected on grounds of R_F.

6. The preparation of an identical 2:4-dinitrophenylhydrazone derivative from compound Y and from synthetic 1-(*o*-carboxyphenylamino)-1-deoxyribulose confirmed the pentose Amadori structure and the identity of compound Y.

We are grateful to Mr G. Morrison for technical assistance, to Mrs B. Segall for her assistance in some of the early experiments and to Dr T. M. Reynolds for suggestions for the preparation and detection of Amadori compounds. Our thanks are due to Professor S. D. Rubbo for his encouragement of this work and to the Australian National Health & Medical Research Council for a grant.

REFERENCES

Aronoff, S. (1956). *Techniques of Radiobiochemistry.* Ames, Iowa: Iowa State College Press.

Aronoff, S. & Vernon, L. (1950). *Arch. Biochem. Biophys.* **28**, 424.

Berger, L. & Lee, J. (1946). *J. org. Chem.* **11**, 75.

Borenfreund, E. & Dische, Z. (1957). *Arch. Biochem. Biophys.* **67**, 239.

Bratton, A. C. & Marshall, E. K. (1939). *J. biol. Chem.* **128**, 537.

Davis, B. D. & Mingioli, E. S. (1950). *J. Bact.* **60**, 17.

Gibson, F., Doy, C. H. & Segall, S. B. (1958). *Nature, Lond.*, **181**, 549.

Gibson, F., Jones, M. J. & Teltscher, H. (1956). *Biochem. J.* **64**, 132.

Hodge, J. E. (1955). *Advanc. Carbohyd. Chem.* **10**, 169.
Inoue, Y., Onodera, K. & Kitaoka, S. (1951–52). *J. Agr. chem. Soc. Japan*, **25**, 291. Cited by Ellis, G. P. & Honeyman, J. (1955). *Advanc. Carbohydr. Chem.* **10**, 139.
Johanson, R. (1953). *Nature, Lond.*, **172**, 956.
Kun, E. & Garcia-Hernandez, M. (1957). *Biochim. biophys. Acta*, **23**, 181.
Lingens, F., Burkhardt, H. J. & Hellmann, H. (1957). *Z. Naturf.* **12***b*, 493.
Mason, M. & Berg, C. P. (1951). *J. biol. Chem.* **188**, 783.
Partridge, C. W. H., Bonner, D. M. & Yanofsky, C. (1952). *J. biol. Chem.* **194**, 269.
Sannié, C. (1948). *C.R. Acad. Sci., Paris*, **226**, 182. Cited by Ellis, G. P. & Honeyman, J. (1955). *Advanc. Carbohydr. Chem.* **10**, 139.
Tracey, M. V. (1950). *Biochem. J.* **47**, 433.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Yanofsky, C. (1956). *J. biol. Chem.* **223**, 171.

Intermolecular and Intramolecular Reactions of Human-Serum Albumin after its X-Irradiation in Aqueous Solution

By D. ROSEN
Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, S.W. 3

(*Received* 15 *December* 1958)

The work detailed below deals with changes found in the ultraviolet-absorption spectra of aqueous solutions of human-serum albumin after X-irradiation at concentrations where the action of the radiation has been shown to be entirely indirect (Rosen, Alexander & Brohult, 1957). This matter has been variously studied in the past 30 years (Spiegel-Adolf & Krumpel, 1927, 1929; review by Arnow, 1936; Barron & Finkelstein, 1952). The purpose of the present paper is threefold: to demonstrate the important part in the absorption spectra of irradiated solutions played by light scattered from aggregated protein; to show how pH affects the aggregation and consequent light-scattering; and from these results to show how irradiation produces both intermolecular and intramolecular effects on the protein, in a proportion varying with circumstances. Although it is known that serum albumin and other proteins in solution are aggregated by ionizing radiation (Carroll, Mitchell & Callanan, 1952; Barron & Finkelstein, 1952; Rosen *et al.* 1957), the fact that this leads to a greatly enhanced scattering of light has been mentioned only by Passynskii (1955) and by Pavlovskaya & Passynskii (1957). It has been considered, however, in a study of ultraviolet irradiation of proteins (Claesson, 1956).

MATERIALS AND METHODS

Chemical reagents. Freeze-dried human-serum albumin was obtained by courtesy of Dr M. E. Mackay from a pharmaceutical preparation made at the Lister Institute, London; it was pure on examination by electrophoresis and by ultracentrifuge. Water used was redistilled in an all-glass apparatus. Other reagents were of the highest purity available commercially. All glassware before use was washed in chromic acid, very thoroughly rinsed with tap water and distilled water and then oven-dried. All solutions were prepared on the day of use and were irradiated while open to the air. The concentrations indicated below are with respect to the protein as supplied. The moisture content of the material was 6–8%, but absolute determinations of this figure and of the final concentrations of protein were not attempted.

Radiation source and its calibration. The X-ray machine used was a Newton KX 10 run at 140 kv and 5 mA with no added filtration (half-value layer 2·86 mm. aluminium), run to give a dose rate between 3000 and 3500 rads/min. (The rad is the unit of absorbed dose, 100 ergs/g.) The output of the machine was calibrated chemically, by the oxidation of ferrous sulphate solution, with the solution occupying the same position and volume as the protein solution irradiated later. The G value (number of molecules changed per 100 ev of energy deposited by the radiation in the solution) was taken as 15·6 (Lefort, 1958).

Spectrophotometry and light-scattering. Absorption spectra were measured on a Unicam SP. 500 spectrophotometer in 1 cm. quartz cells. The relation between the weight-average molecular weight, M_w, and the light-scattering effect in the transmitted light is given by a modification, due to Debye and Cachin, of the standard Rayleigh formula (see Stacey, 1956). At a concentration c (g./ml.), the turbidity τ (that part of the absorption coefficient due solely to light-scattering) is given by

$$\frac{H_c}{\tau} = \frac{1}{M_w} \cdot \frac{1}{T},$$

where

$$H = \frac{32\pi^3 n_0^2 (dn/dc)^2}{3\lambda^4 N}$$

and

$$\frac{1}{T} = 1 + \frac{16\pi^2 r^4 n^4 n_0^2}{3\lambda^4}.$$

Here n_0 and n are the refractive indices of the solvent and solution, N is Avogadro's number, λ is the wavelength (*in vacuo*) of the light in which measurement is being made, and r is the radius of gyration of a molecule of the solute.

In the present work, the correction factor T did not depart significantly from unity. A direct measurement of light scattered was made with the apparatus described by Stacey (1956) at λ 4860 Å.

RESULTS

Irradiation of isoelectric solutions. For a 0·1 % solution of salt-free human-serum albumin at its isoelectric point (pH 5·4) the ultraviolet-absorption spectrum is shown in Fig. 1 (*a*) (curve *A*). After irradiation (20 500 rads) the absorption spectrum was that shown in Fig. 1 (*a*) (curve *B*). From the two curves, the increase (Δ) in absorption at each wavelength was obtained, and Fig. 1 (*b*) gives a plot of $\lambda^4\Delta$. It is evident that, except for a slight irregularity at 295–300 mμ, the value of $\lambda^4\Delta$ is constant over the range covered. Such a result is strongly indicative of Rayleigh scattering of the light by aggregated protein. To test this point, direct measurements were made of the light scattered by the protein solutions. Because the irradiated solutions contained a small amount of material of very high molecular weight, the errors of measurement were particularly large. However, within the limits of the standard error, the average molecular weight from direct measurement of light-scattering and from interpretation of the change in absorption spectrum showed agreement: a typical result is given in Table 1. Previous examination by ultracentrifuge had shown (Rosen *et al.* 1957) that the dose given would have caused about 47 % of the protein to aggregate into complexes of two, three, four, etc., protein molecules. The present measurements of average molecular weights gave results entirely compatible with the earlier ultracentrifuge measurements.

Hence it was taken as a working rule that, when the quantity $\lambda^4\Delta$ was constant (within the range of experimental error of up to 15 %) over all or nearly all the available wavelength range, then it could be taken as an indication of aggregation of the protein molecules and as a measure of average molecular weight, provided that there was general

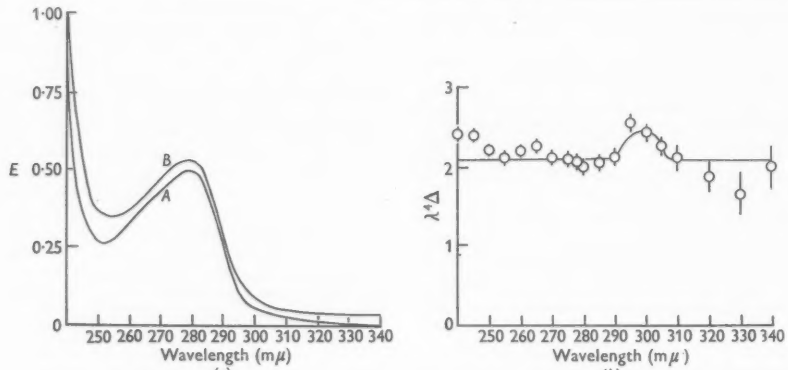


Fig. 1. Change in ultraviolet-absorption spectrum of 0·1 % salt-free isoelectric solution of human-serum albumin after X-irradiation. (*a*) Curve *A*: spectrum before irradiation; curve *B*: spectrum after a dose of 20 500 rads of 140 kv X-rays. (*b*) Analysis for Rayleigh scattering where Δ is the difference in extinction between curves *A* and *B* at wavelength λ. The ordinate is in units of $10^{-8} \times$ (wavelength in mμ)$^4 \times$ (measured change in extinction).

Table 1. *Increase of weight-average molecular weight of human-serum albumin when X-irradiated in aqueous solution*

Concn. (%)	pH	Dose of X-rays (rads)	Molecular wt. By light-scattering	Molecular wt. By u.v. absorption
0·1	5·4	—	94 000 ± 2 900	70 000 (assumed)*
		20 000	250 000 ± 50 000	308 000 ± 29 000
0·1	3·6	—	78 500 + 7 100	—
		40 000	170 000 + 30 000	—

* The amount of scattering was too small for the molecular weight to be determined by the appearance of the u.v.-absorption spectrum, but the acknowledged molecular weight of the protein was assumed for computing the physical constants involved.

supporting evidence of aggregation from other methods, e.g. sedimentation or orthodox light-scattering measurements. It may be noted that if Δ is known to be produced solely by scattering in the treated solution, then for any particular amount of scattering material Δ will be independent of the concentration of non-scattering material in the original untreated solution. And specifically, a particular dose of radiation, acting indirectly and causing a determinable amount of protein to aggregate (Rosen *et al.* 1957) will lead to a value of Δ for each wavelength independent of the initial protein concentration.

As irradiation of the solution was continued, aggregation of the protein proceeded until the solution became cloudy and the protein was precipitated. In the later stages, the scattering of light was so great that very little passed through the solution. In ultraviolet light, the increase in apparent absorption was initially proportional to the dose, but it became more and more rapid at high doses.

Irradiation of non-isoelectric solutions. Solutions of the protein were brought to various values of pH by the addition of appropriate amounts of hydrochloric acid or sodium hydroxide solution. In Fig. 2 are shown graphs of $\lambda^4\Delta$ against λ (cf. Fig. 1*b*) for two solutions of human-serum albumin X-irradiated at values of pH away from the isoelectric point. In these circumstances Δ was no longer inversely proportional to λ^4. There was thus no immediate indication of an effect of light-scattering, since Δ might have been entirely the product of a true change in absorption or might have been compounded partly of this and partly of a scattering component. There was, however, a maximum in the curve at ~ 299 mμ, as found with isoelectric solutions; yet although they had received a higher dose than the isoelectric solution illustrated in Fig. 1, both the solutions shown in Fig. 2 had substantially lower values of $\lambda^4\Delta$ and these values were not sufficiently constant with λ for an average to be determined.

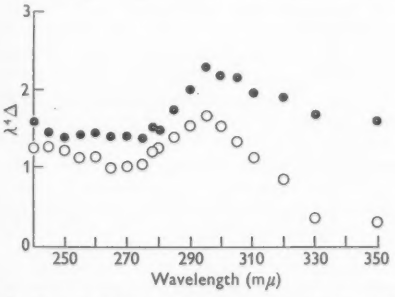


Fig. 2. Plot of $\lambda^4\Delta$–λ (cf. legend to Fig. 1*b*) for 0·13% solutions of human-serum albumin at values of pH away from the isoelectric point and irradiated with a dose of 24 600 rads of 140 kv X-rays: ○, pH 3·8; ●, pH 10·2.

But although Fig. 2 does not demonstrate that part of the increased extinction of the solutions was due to increased light-scattering, it does not preclude the possibility. Ultracentrifuge examination showed aggregation in irradiated non-isoelectric solutions similar to that in isoelectric ones (Rosen *et al.* 1957); and direct measurement of light-scattering showed an increase on irradiation from which average molecular weights could be calculated (Table 1). On moving away from the isoelectric point, the dose of radiation required to produce any particular amount of aggregation (determined by ultracentrifuge measurement of the

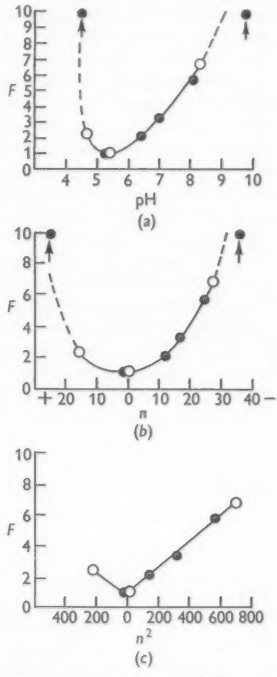


Fig. 3. The aggregation and precipitation of X-irradiated solutions of human-serum albumin. In each case the ordinate, *F*, is the factor by which the dose must be increased over that dose required at the isoelectric point to produce a given amount of aggregation or precipitation. (*a*) Abscissa: pH. (*b*) Abscissa: *n*, the number of positive or negative charges on protein molecules, determined separately from the titration curve. (*c*) Abscissa: n^2. ○, Determined from sedimentation diagram; ●, determined from the onset of visible precipitation. The arrows indicate points lying outside the scales.

amount of material sedimenting normally) was greater by a factor F than the dose required at the isoelectric point. The same was true of the dose required to produce precipitation (which occurred quite suddenly at a dose determinable with an accuracy of about 5%). The factor F was found to be proportional to the square of the molecular charge, as calculated from the acid–base titration curve in a separate measurement (Fig. 3), and hence proportional to the collision frequency of the protein molecules.

Addition of inorganic salts (NaCl, Na_2HPO_4–NaH_2PO_4 mixtures, and NaF) in up to half-molar concentrations enhanced the effect of changing the pH.

Thus irradiation of non-isoelectric solutions caused aggregation or intermolecular reaction (revealed by ultracentrifuge examination and light-scattering measurements) to an extent depending on the pH; and also a considerably greater intramolecular change (revealed by changes in absorption spectrum) than occurred in irradiated isoelectric solutions.

Effect of change of pH *after irradiation.* Addition of hydrochloric acid to an irradiated solution after irradiation to lower the pH to between 2·0 and 2·5 had a profound effect within an hour. Firstly, it dispersed the aggregates formed on irradiation. This could be seen by eye in solutions irradiated heavily enough for a precipitate to have appeared. In less heavily irradiated solutions the same effect could be seen on examination in the ultracentrifuge.

The effect of breaking down the aggregates could also be seen in the decrease of light scattered and the consequent change in the ultraviolet-absorption spectrum. This is shown in Fig. 4.

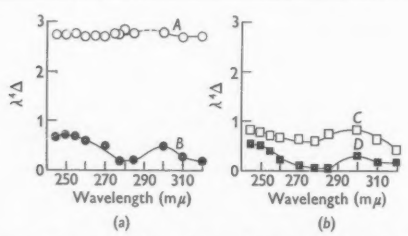


Fig. 4. (*a*) Plot of $\lambda^4\Delta$–λ (cf. legend to Fig. 1*b*) for a 0·13% solution of human-serum albumin at pH 5·4 irradiated with 15 000 rads of 140 kv X-rays and then diluted (1:1) with water (curve *A*) and 0·01N-HCl (curve *B*). For computing curve *B*, Δ was taken with respect to the unirradiated solution similarly diluted with acid. (*b*) Plot of $\lambda^4\Delta$–λ for a 0·13% solution of human-serum albumin at pH 3·9, treated otherwise as the solution in (*a*). (No measurements were made between 285 and 300 mμ, but the dotted part of curve *A* has been drawn to accord with Fig. 1*b*.)

A solution irradiated at the isoelectric point had a change in extinction almost entirely due to Rayleigh scattering (as shown by the $\lambda^4\Delta$ plot in Fig. 4*a*, curve *A*; compare Fig. 1). After the pH had been lowered to 2·35 by the addition of hydrochloric acid, the change in extinction was greatly reduced and was of a different character (Fig. 4*a*, curve *B*) resembling the change produced when a solution was irradiated away from the isoelectric point; the plot of $\lambda^4\Delta$ against λ shows a sinuosity which did not exist at the isoelectric point. Hence, apart from the breakdown of the aggregates, some further change took place (compared with a similarly treated control on which the addition of hydrochloric acid had no effect) which affected the absorbing groups of the protein.

When a slightly acidified solution (pH 3·90) was irradiated, the resulting plot (Fig. 4*b*, curve *C*) of $\lambda^4\Delta$ against λ is similar to that of the solution irradiated at the isoelectric point and then acidified. However, it is rather flatter, and also the presence of some aggregated protein, known to exist from other studies (see the preceding section), can be inferred from the height of the curve above the base line. When this solution was further acidified (to pH 2·35) it yielded a plot of $\lambda^4\Delta$ (Fig. 4*b*, curve *D*) which shows a reduced change in extinction (compared with a similarly treated control) but an enhanced internal change as revealed by the sinuosity of the curve.

The final acidified solutions of the isoelectric and non-isoelectric preparations (curves *B* and *D*) suffered, within experimental error, equal amounts of internal intramolecular change. A small parallel upward displacement of curve *B* relative to curve *D* suggests that a residue of aggregated protein was left in the solution which was initially at the isoelectric point.

Addition of alkali to the solutions after irradiation produced effects similar to the addition of acid.

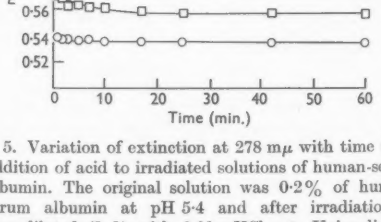


Fig. 5. Variation of extinction at 278 mμ with time after addition of acid to irradiated solutions of human-serum albumin. The original solution was 0·2% of human-serum albumin at pH 5·4 and after irradiation it was diluted (1:1) with 0·01N-HCl. ○, Unirradiated; □, 56 000 rads; △, 84 000 rads.

But interpretation of the absorption spectra in these conditions was complicated and made uncertain by the change in extinction coefficient of the aromatic amino acids with ionization at high pH. Back-titration from extreme values of pH towards the isoelectric point did not allow reformation of the aggregated protein nor did it remove the changes in absorption spectra ascribed to intramolecular changes.

The time taken for the changes caused by the addition of acid to an irradiated solution to manifest themselves fully was about half an hour (Fig. 5). The corresponding changes produced by the addition of alkali took somewhat longer to complete (several hours).

Addition of urea after irradiation. Addition of urea to irradiated solutions of human-serum albumin to give a final concentration of 4–6 M-urea, and left standing for 2–15 hr., failed to disperse precipitated protein and caused no change in the sedimentation diagram of solutions examined in the ultracentrifuge. Also, within the limits of experimental error, there was no change in the ultraviolet-absorption spectrum when a solution of urea and irradiated protein (measured with respect to a solution of urea and unirradiated protein) was compared with irradiated protein without urea (measured with respect to unirradiated protein without urea).

DISCUSSION

The action of ionizing radiations on serum albumin in dilute aqueous solutions is effected by free radicals produced in the radiolysis of water (Barron & Finkelstein, 1952; Rosen *et al.* 1957). The results presented here show that the reaction between the free radicals and a protein molecule can lead either to a partly reversible intermolecular aggregation of molecules, or to an irreversible intramolecular change in the reacting protein. The extent to which the intermolecular and intramolecular effects separately occur depends on the pH and ionic strength of the solution, and the occurrence of the intermolecular effect appears to be determined by the collision frequency of protein molecules in the solution.

The situation can be explained by the hypothesis that the effect produced by X-rays on proteins in solution is a two-stage process. The first stage is the reaction between the protein molecule and a free radical formed in the radiolysis of the solvent, this yielding a reactive state of the protein molecule. The second stage is the reaction of this compound either inter- or intra-molecularly. It must be supposed that when both paths of reaction are possible, the intermolecular reaction is much the more probable at the concentrations used in the present study; this reaction, however, can take place only during the moment of contact in a collison. When the collision frequency is reduced (by charged molecules repelling one another), the intramolecular reaction becomes relatively more probable. A similar hypothesis of a two-stage mechanism has been put forward since the present study was completed, by Eidus, Kondakova & Otarova (1958), to explain the effects of oxygen in the irradiation of solutions of myosin.

The absorption spectra give very little indication of the chemical nature of either the intermediate or final products of the denatured protein. Barron & Finkelstein (1952) suggested that the changes at wavelengths greater than 240 mμ in the spectrum of serum albumin after irradiation were entirely due to oxidation of tyrosine, giving dihydroxyphenylalanine. The present results show that this is not the case and even the small part of the increase in absorption which is due to an intramolecular change may not be due to a change in tyrosine. The difference between the absorption spectra of tyrosine and either dihydroxyphenylalanine or irradiated tyrosine shows a strong sharp peak at 285 mμ (D. Rosen, unpublished work), whereas in the irradiated protein a small peak, revealed only by mathematical manipulation, occurs some distance away at 295–300 mμ.

SUMMARY

1. The effect of 140 kv X-radiation on aqueous solutions of human-serum albumin has been studied by ultraviolet-absorption spectroscopy, supplemented by light-scattering measurements and ultracentrifuge examination.

2. It is shown that, depending on the conditions of pH and ionic strength, the radiation can produce either or both of two effects: a partly reversible intermolecular effect of aggregation, and an irreversible intramolecular effect which can be detected spectroscopically.

3. The intermolecular aggregation causes light-scattering, which affects the absorption coefficient in spectroscopic measurements. A method of correcting for this is used which, in isoelectric solutions where the radiation effect is almost entirely intermolecular, yields a value of the average molecular weight in quantitative agreement with direct light-scattering measurement.

4. A two-stage process is proposed for the changes occurring in proteins in these conditions.

I am particularly grateful to Dr P. Alexander for his constant interest and encouragement.

The work has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for

Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

Arnow, L. E. (1936). *Physiol. Rev.* **16**, 671.
Barron, E. S. G. & Finkelstein, P. (1952). *Arch. Biochem. Biophys.* **41**, 212.
Carroll, W. R., Mitchell, E. R. & Callanan, M. J. (1952). *Arch. Biochem. Biophys.* **39**, 232.
Claesson, I. M. (1956). *Ark. Kemi*, **10**, 1.
Eidus, L. Kh., Kondakova, N. V. & Otarova, G. K. (1958). *Biofizika*, **3**, 215.
Lefort, M. (1958). *Advanc. phys. Chem.* **9**, 123.
Passynskii, A. G. (1955). *Conf. Acad, Sci. U.S.S.R. (Session Div. Biol. Sci.): Peaceful Uses of Atomic Energy*, p. 69. Cited in *Chem. Abstr.* (1955), **49**, 16002i.
Pavlovskaya, T. E. & Passynskii, A. G. (1957). *Biokhimiya*, **22**, 266.
Rosen, D., Alexander, P. & Brohult, S. (1957). *Arch. Biochem. Biophys.* **70**, 266.
Spiegel-Adolf, M. & Krumpel, O. (1927). *Biochem. Z.* **190**, 28.
Spiegel-Adolf, M. & Krumpel, O. (1929). *Biochem. Z.* **208**, 45.
Stacey, K. A. (1956). *Light Scattering in Physical Chemistry*. London: Butterworth and Co. Ltd.

Thetin–Homocysteine Transmethylase

THE DISTRIBUTION OF THE ENZYME, STUDIED WITH THE AID OF TRIMETHYLSULPHONIUM CHLORIDE AS SUBSTRATE

By G. A. MAW*
Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E. 1

(*Received* 19 *January* 1959)

Thetin–homocysteine transmethylase, which catalyses the transfer of a methyl group from dimethylthetin or dimethyl-β-propiothetin to homocysteine, was first identified in rat, pig and guinea-pig liver and kidney by Dubnoff & Borsook (1948*a*, *b*). Kensler & Langemann (1954) detected the transmethylase in the livers of a number of other mammalian species, including man, and it was subsequently found in horse liver (Durell & Anderson, 1956) and in pigeon liver (Sloane, Boggiano, Coulomb & Hutchings, 1956). Thoai & Robin (1956) also showed the presence of the enzyme in turtle and mullet liver and produced evidence for its occurrence in several invertebrates. According to Schlenk & DePalma (1956), the yeast *Torulopsis utilis* is unable to utilize the above-mentioned thetins as methyl donors for homocysteine and, presumably, does not possess the enzyme. Shapiro (1958) has recently obtained similar findings for *Aerobacter aerogenes*, *Saccharomyces cerevisiae* and some strains of *Escherichia coli*.

In assays of thetin–homocysteine transmethylase carried out previously by other workers, the activity of the enzyme has been expressed in terms of the amount of methionine formed in a given time, the product being determined colorimetrically. It is not possible to compare these published findings on a quantitative basis, since in most cases optimum substrate concentrations were not employed, and no attention appears to have been paid to whether methionine formation was linear with respect to time or whether the experimental conditions chosen gave a linear relationship between enzyme activity and amount of tissue preparation.

In this paper a manometric method has been used for the study of the transmethylase. This method measures enzyme activity in terms of the rate of carbon dioxide formation from a bicarbonate medium, since the methylation reaction involves the formation of acid (Maw, 1955). With either dimethylthetin chloride or dimethyl-β-propiothetin chloride as methyl donor, it was found that neither the reaction rates nor activity/concentration relationships obtained with rat-liver thetin–homocysteine transmethylase were linear (Maw, 1956). However, it was shown (Maw, 1958) that trimethylsulphonium chloride acts as a methyl donor for the enzyme and, with this compound as substrate, reaction rates as well as activity/concentration relationships were linear. Trimethylsulphonium chloride was therefore suggested as a possible substrate for the assay of the transmethylase.

Further evidence is now presented that trimethylsulphonium chloride can act as a substrate for the enzyme and can be used in its assay. This compound has been employed in a manometric study of the transmethylase in various tissues of the rat, mouse and guinea pig, and in a survey of its occurrence in twenty-four species of plant and

* Present address: Chemist's Laboratory, Arthur Guinness, Son & Co. (Dublin) Ltd., St James's Gate, Dublin, Eire.

animal. The transmethylase has been detected in significant amounts only in the higher animals tested, where it is located mainly in the liver. This is in qualitative agreement with the findings of other workers, referred to above. The enzyme appears to be present only in negligible quantities, if at all, in the green plants, moulds and invertebrates examined.

MATERIALS AND METHODS

Dimethylthetin chloride, trimethylsulphonium chloride, triethylsulphonium chloride and the other sulphonium salts employed were prepared as described in a previous paper (Maw, 1958). DL-Methionine was demethylated to homocystine with sulphuric acid (Butz & du Vigneaud, 1932) and reduced to homocysteine with sodium in anhydrous NH_3 (Riegel & du Vigneaud, 1935–36).

The various species of animal under study, with the exception of the dog, were killed by a blow on the head and the required tissues were excized and placed in ice. Samples of dog liver were obtained from animals which had been bled to death under *cyclo*propane anaesthesia. Suspensions of the tissues were prepared as soon as possible in 0·02 M-$NaHCO_3$ with a glass homogenizer (Potter & Elvehjem, 1936), and filtered through several layers of muslin. Plant-tissue suspensions were obtained by grinding the tissue with fine sand in 0·02 M-$NaHCO_3$. When moulds were examined, the pellicle was washed with the bicarbonate solution and sliced before being homogenized. Baker's yeast was used in the form of suspensions of the fresh organism and also cell-ruptured preparations were obtained by freezing in solid CO_2, followed by thawing at 37° (Krebs, Gurin & Eggleston, 1952).

In assays of thetin–homocysteine transmethylase, 0·5 ml. of 10% (w/w) tissue suspension in 0·02 M-$NaHCO_3$ was used in the side arm of each Warburg flask, unless otherwise stated. The main compartment of each flask contained DL-homocysteine (0·03 M) and either dimethylthetin (DMT; 0·1 M) or trimethylsulphonium chloride (TMS; 0·2 M). In other experiments, such as studies of the effects of inhibitors, the methyl donors were added at lower concentrations, as described in subsequent sections. The substrates and other substances used were prepared in 0·02 M-$NaHCO_3$, as described previously (Maw, 1958). The total fluid volume in each flask was 2·5 ml. Flasks were gassed with $N_2 + CO_2$ (95:5, v/v), and formation of CO_2 was followed for 40–60 min. after mixing of the tissue suspensions with the substrates.

Enzyme activity has been calculated in terms of the initial reaction rate, this being taken as the volume of CO_2 liberated during the 10 min. period commencing 5 min. after the enzyme has been mixed with the substrates. The rate obtained was then expressed as a metabolic quotient, Q_{CO_2}, i.e. μl. of CO_2/mg. dry wt. of tissue preparation/hr. The use of the Q notation is strictly of value only when TMS has been used as the methyl donor, i.e. under conditions which yield a linear enzyme activity/concentration relationship. However, rates obtained with DMT as substrate have also been expressed in this way because, in almost all the experiments reported, enzyme activities have been compared at a fixed tissue concentration.

RESULTS

Trimethylsulphonium chloride as a substrate

Tissue preparations which give rise to carbon dioxide formation in the presence of homocysteine and DMT behave similarly when the thetin is replaced by TMS. Although this may be taken to indicate that TMS is a substrate for thetin–homocysteine transmethylase, there is also the possibility that the compound is being demethylated by a second transmethylase present. A number of experiments have therefore been carried out to determine whether or not DMT and TMS are acted on by the same enzyme.

(i) *Parallelism of substrate activity.* The methylation of homocysteine by the two sulphonium salts has been studied in the presence of various tissue suspensions obtained from a wide range of plants and animals (see Table 2). Whenever carbon dioxide formation was observed with DMT as substrate, there was a concomitant activity detectable with TMS. In no case was transmethylase activity demonstrable with one of the methyl donors and not the other.

(ii) *Distribution of transmethylase activity in rat-liver preparations.* Rat-liver suspensions were subjected to ammonium sulphate fractionation (see Maw, 1956). Four fractions were obtained, namely, material precipitated at 0–0·35, 0·35–0·45 and 0·45–0·55 saturation and the final supernatant fluid. These fractions were dialysed against running tap water for 15 hr. at room temperature and then centrifuged at 1600 *g* for 10 min. The precipitates

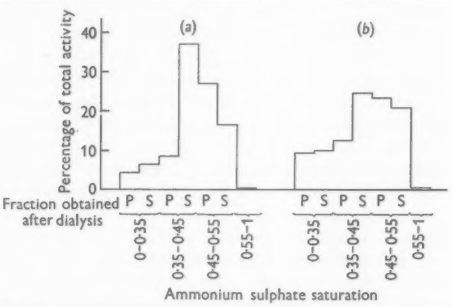


Fig. 1. (*a*) Distribution of thetin–homocysteine transmethylase activity in fractions of rat liver obtained by ammonium sulphate precipitation. Liver suspension, 10% (w/w); substrates: DL-homocysteine (0·03 M), DMT (0·1 M); 0·02 M-bicarbonate medium; flasks were gassed with $N_2 + CO_2$ (95:5, v/v); temp. 37·1°; P, precipitate; S, supernatant fluid. (*b*) Distribution of thetin–homocysteine transmethylase activity in fractions of rat liver obtained by ammonium sulphate precipitation. Substrates: DL-homocysteine (0·03 M), TMS (0·2 M). Other details were as given for (*a*).

from each fraction were suspended in 0·02M-$NaHCO_3$, the supernatant portions made 0·02M with respect to $NaHCO_3$ and all samples were tested for transmethylase activity, DMT and TMS being used as substrates. Figs. 1*a* and 1*b* show the activities of the various fractions obtained with the two substrates, expressed as percentages of the total activity present, and it will be seen that TMS gives an activity-distribution pattern similar to that obtained with DMT.

(iii) *Transmethylase activity with mixed substrates.* If carbon dioxide production occurring in the presence of DMT and TMS is brought about by two separate transmethylases, then when the two compounds are added together to enzyme preparations there should be a summation of carbon dioxide formation. If, on the other hand, only one enzyme is involved, there should be competition between the substrates for the enzyme, and the observed reaction rate obtained with the mixed substrates should not be greater than the rates obtained with either substrate alone (the mixed substrate reaction rate should, in fact, be somewhere in between these two values, depending on the relative affinities of the two substrates for the enzyme). That one enzyme only is operating is suggested by the results given in Figs. 2*a* and 2*b*. These were obtained with a mouse-liver suspension and an eel-liver suspension respectively. They show that no summation of carbon dioxide production occurred when the mixed substrates were used. Furthermore, although the more active substrate was different in each case, the combined rate was in both cases closer to the rate for DMT alone, indicating that the enzyme has a greater affinity for the thetin. Findings confirming this lack of any summation of reaction rates have also been obtained with liver homogenates from the rat, pigeon and guinea pig. With some of the enzyme preparations used, e.g. rat- and mouse-liver homogenates, TMS was a markedly less effective substrate than the thetin, and in order to provide the maximum opportunity for competitive effects to make themselves apparent, the concentration of DMT was reduced well below the optimum value.

(iv) *Effects of structural inhibitors and inorganic salts.* A similarity has been observed between the types of compound which inhibit carbon dioxide formation with DMT as methyl donor and those which inhibit in the presence of TMS. As would be expected, the effects were more marked with the latter substrate, on account of its lower affinity for the transmethylase. Inhibitions produced by the various compounds used were small or negligible with an optimum concentration of DMT, but became more apparent when lower concentrations of the thetin were employed. Table 1 shows the effects of a series of inhibitors on the activity of the mouse-liver transmethylase. The compounds have been arranged in order of their effectiveness in the presence of 0·01M-DMT, and it will be seen that this corresponds closely to the order of their effectiveness when 0·1M-TMS was the substrate. Parallel results have been obtained with guinea-

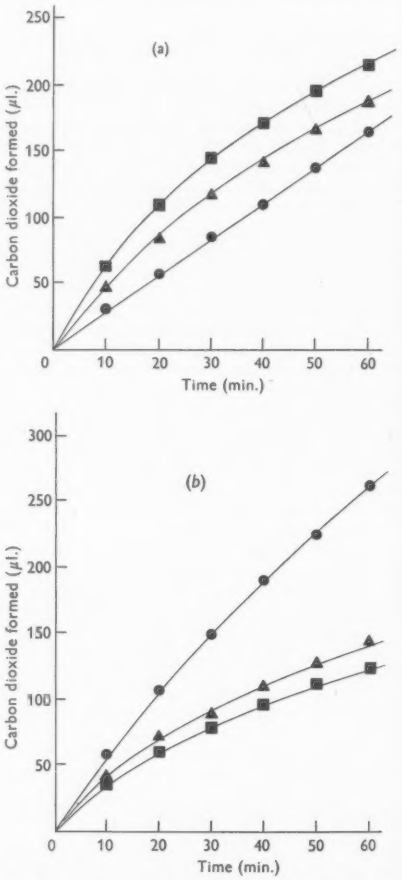


Fig. 2. (*a*) Formation of carbon dioxide by a mouse-liver suspension, DMT and TMS being used as methyl donors. Tissue suspension, 2% (w/w); DL-homocysteine (0·03M), DMT (0·025M), TMS (0·2M); 0·02M-bicarbonate medium; flasks were gassed with N_2+CO_2 (95:5, v/v); temp. 37·1°. ■, DMT alone as methyl donor; ●, TMS alone as methyl donor; ▲, DMT+TMS as methyl donor. (*b*) Formation of carbon dioxide by an eel-liver suspension, DMT and TMS being used as methyl donors. Tissue suspension, 1% (w/w); DL-homocysteine (0·03M), DMT (0·1M), TMS (0·2M). Other details were as given for (*a*).

Table 1. *Inhibition of mouse-liver thetin–homocysteine transmethylase by various sulphonium and quaternary ammonium halides*

Mouse-liver suspensions, 2% (w/w); DL-homocysteine (0·03M); dimethylthetin chloride (DMT; 0·01M); trimethylsulphonium chloride (TMS; 0·1M); 0·02M-sodium bicarbonate medium; flasks were gassed with N_2+CO_2 (95:5, v/v); temp. 37·1°.

Inhibitor (0·1M)	Percentage inhibition With DMT	Percentage inhibition With TMS
Betaine chloride	70·3	86·0
Triethylsulphonium chloride	34·9	58·0
Sulphocholine iodide	31·8	26·7
Diethylthetin chloride	25·3	41·7
Choline chloride	23·6	41·2
Tetramethylammonium bromide	14·5	24·4
Ammonium chloride	8·9	3·6

pig-liver homogenates. By the method of Lineweaver & Burk (1934), the inhibitions produced by betaine chloride, tetramethylammonium bromide and triethylsulphonium chloride have been shown to be strictly competitive in character.

Mercuric ions markedly inhibit the rat-liver transmethylase in the presence of DMT (Maw, 1958), and this is also true when TMS is the methyl donor. It is also significant that bromide and iodide ions (as sodium salts) produce a slight increase in transmethylase activity, which is apparent when either sulphonium compound is used as substrate. Thus in the presence of 0·1M-TMS or -DMT, 0·1M-sodium iodide increases the activity of the transmethylase of mouse and guinea-pig liver by approximately 25%.

In a single experiment, determinations of the inhibitor constant, K_i, for betaine chloride were carried out with the mouse-liver transmethylase, DMT and TMS being used separately as substrates (Lineweaver & Burk, 1934). K_i values so obtained should be equal if the substrates employed are acted upon by the same enzyme (see Dixon & Webb, 1958). They were, in fact, found to be comparable in magnitude, namely $6{\cdot}4\times10^{-2}$ with DMT as substrate, and $3{\cdot}7\times10^{-2}$ in the presence of TMS.

The various findings outlined above are considered to point strongly to the existence of a single transmethylase utilizing both DMT and TMS as methyl donors, but having a greater affinity for the former compound.

Assay of the transmethylase

With TMS as the methyl donor, a brief examination has been made of carbon dioxide formation corresponding to thetin–homocysteine transmethylase activity in various tissues. With 0·1- or 0·2M-TMS, carbon dioxide evolution was generally strictly linear for 1 hr. at least, as found previously for preparations of the rat-liver enzyme (Maw, 1958), although for some tissues, notably pigeon liver, there was occasionally a slight falling off in rate after about 30 min. It was therefore possible to make accurate determinations of initial reaction rates.

Plots of initial reaction rate against the volume of tissue suspension used have been carried out for liver homogenates from eight animal species, namely the dog, eel, goldfish, guinea pig, mouse, pigeon, rabbit and rat. In every case a strictly linear relationship was obtained with tissue concentrations ranging up to 2% (w/w) of the reaction mixture. It is therefore considered that, provided the amounts of tissue taken do not greatly exceed this value, the manometric procedure embodying the use of TMS as substrate may be reliably employed for the assay of thetin–homocysteine transmethylase.

Distribution of the transmethylase

Table 2 gives the results of a survey of thetin–homocysteine transmethylase activity in 24 plant and animal species, each value reported being the average of at least three separate determinations. The choice of substrate concentrations, namely 0·1M-DMT and 0·2M-TMS, was based on previous experiments with the rat-liver transmethylase. DMT was originally used only for the qualitative examination of the enzyme in the various source materials, but for comparative purposes activities obtained with this substrate have been expressed as Q_{CO_2} values.

Under the conditions of the transmethylase assay there was some carbon dioxide formation with homogenates from the majority of the green plants, moulds and samples of baker's yeast examined. This was small, however, and comparable with that observed in the absence of substrates. Moreover, it fell off sharply after 30 min. and in no way resembled the steady carbon dioxide production obtained with transmethylase preparations from animal liver. It is therefore assumed that transmethylase activity is negligible or absent in these species. Of the four invertebrates studied, only the common shore crab appeared to possess some slight transmethylase activity.

Significant activity was detected in the liver of all the vertebrates examined. Rat liver was a poor source of the enzyme, liver from the mouse, dog and guinea pig being two to three times as active. The most active tissue examined was eel liver, which contrasted markedly with goldfish liver in this respect. The enzyme was definitely present in rat and guinea-pig kidney, although its activity was low. Negligible amounts were found in mouse kidney and in the muscle, spleen and brain of the rat, mouse and guinea pig.

Table 2. *Thetin–homocysteine transmethylase activity of various tissues*

Tissue suspensions, 2% (w/w); DL-homocysteine (0·03M); dimethylthetin chloride (DMT; 0·1M); trimethylsulphonium chloride (TMS; 0·2M); 0·02M-sodium bicarbonate medium; flasks were gassed with $N_2 + CO_2$ (95:5, v/v); temp. 37·1°. Activity is expressed as Q_{CO_2} (μl. of CO_2/mg. dry wt. of tissue/hr.).

Species	Tissue	Q_{CO_2} (DMT)	Q_{CO_2} (TMS)
Rat	Liver	13·2	6·9
	Spleen, muscle, brain	0	0
	Kidney	4·3	2·0
Mouse	Liver	27·7	15·8
	Spleen, muscle, brain	0	0
	Kidney	0·9	0·5
Guinea pig	Liver	9·2	15·5
	Spleen, muscle, brain	0	0
	Kidney	2·4	1·8
Dog	Liver	14·5	20·4
Rabbit	Liver	17·0	11·2
Chicken	Liver	6·1	9·3
Pigeon	Liver	7·9	8·8
Goldfish	Liver	8·4	8·1
Eel	Liver*	25·5	65·6
Frog	Liver	11·7	15·3
Tortoise	Liver	2·9	2·8
Locust (adult)	Hepatic caecae, fat body, whole viscera	0	0
Cockroach	Whole viscera	0	0
Common mussel	Whole viscera	0	0
Common shore crab	Liver	1·6	0·4
	Gonads	0·8	0·5
	Gills	2·8	1·2
French kidney bean	Bean, leaf, root	0	0
Antirrhinum	Leaf	0	0
Lupin	Leaf, root	0	0
Cabbage	Heart, outer leaves, root	0	0
Aspergillus niger	Pellicle	0	0
Penicillium chrysogenum	Pellicle	1·4	0·9
Scopulariopsis brevicaulis	Pellicle	0·5	0·5
Neurospora crassa (three strains)	Mycelia	1	1
Baker's yeast	Whole cells, broken cells	0	0

* Tissue suspension (1%, w/w) was used.

It will be noted that, for the liver of some species, e.g. the rat, mouse and rabbit, initial rates were greater with 0·1M-DMT as substrate than with 0·2M-TMS, whereas with liver from the guinea pig, dog, eel, etc., the reverse was the case. This apparent variation in substrate activity is possibly due to species differences in the shapes of the activity/substrate concentration curves for the two methyl donors.

DISCUSSION

This work, coupled with that of previous investigators, has revealed the presence of thetin–homocysteine transmethylase in the livers of a wide range of vertebrates. The thetin transmethylase activity of the various bacteria, moulds, yeasts and green plants tested has, on the other hand, been found to be negligible. Thoai & Robin (1956) detected the enzyme in tissues from certain invertebrates, including the hemichordate *Glossobalanus*, the sea-urchin *Sphaerechinus* and the brittle star *Ophiothrix*, but found it absent from the thorn-backed crab *Maia* and the squid *Sepia*. This may be compared with the findings given here for the locust, cockroach, common mussel and common shore crab. The apparently variable occurrence of the transmethylase among marine invertebrates may possibly be linked with their feeding habits, for dimethyl-β-propiothetin, which

is a good substrate for the transmethylase, is known to be present in various marine algae (Challenger & Simpson, 1948; Bywood & Challenger, 1953), and is therefore a likely constituent of their diet.

The thetin transmethylase appears to be a characteristic component of animal liver, but its role in metabolism is not an obvious one, despite the fact that it is present in this tissue in appreciable amounts, and in the horse, for example, accounts for 1% of the total liver proteins (Durell, Anderson & Cantoni, 1957). It is unlikely to be concerned solely with the demethylation of simple thetins, such as dimethylthetin, since these compounds are not usually constituents of an animal diet, and they are apparently not present in detectable amounts in animal tissues (Dubnoff & Borsook, 1948*b*). The transmethylase could, however, participate in the catabolism of *S*-methylmethionine, which is a substrate for the rat-liver enzyme (Maw, 1958), and which also occurs widely in plants (Challenger & Hayward, 1954; McRorie *et al.* 1954). According to Durell *et al.* (1957), the enzyme does not act on *S*-adenosylmethionine and, furthermore, it is distinct from the adenosylmethionine–homocysteine transmethylase of bacteria (Shapiro, 1958) and from the transmethylases requiring as methyl acceptors compounds other than homocysteine, e.g. glycocyamine and nicotinamide.

One possibility is that it normally acts on some other intermediate in transmethylation, such as betaine. Purified thetin transmethylase preparations from horse liver have been shown to possess betaine transmethylase activity (Durell *et al.* 1957), and in the present studies betaine was found to be a potent competitive inhibitor of the transmethylase, which would be explained if betaine were acting as a substrate. It is not possible to test this suggestion by means of the manometric method employed here, but direct determination of the amount of methionine formed from dimethylthetin chloride and betaine by the purified enzyme with the mixed substrate technique, together with a further study of the kinetics of the transmethylase, would be of value in this respect.

Recent work on betaine–homocysteine transmethylase by Ericson (1958*a*) has shown that highly purified preparations of this enzyme from pig liver possess marked thetin transmethylase activity. The betaine transmethylase and thetin transmethylase activities of these preparations were destroyed at the same rate by heat treatment and, in addition, both activities were inhibited by dimethylglycine. There are therefore close similarities between the properties of thetin–homocysteine transmethylase and betaine–homocysteine transmethylase. This applies also to their occurrence in tissues, for Ericson (1958*b*) has reported that the betaine transmethylase is present in the liver of vertebrates, but is absent from other organs and from the tissues of invertebrates and plants, a distribution resembling that now found for the thetin transmethylase. These similarities emphasize the desirability of obtaining more detailed comparative information about these two transmethylases, since it is possible that they may even be identical.

SUMMARY

1. Evidence is presented that trimethylsulphonium chloride is a substrate for thetin–homocysteine transmethylase, and may be used in the manometric assay of the enzyme.

2. A survey has been made of the distribution of thetin–homocysteine transmethylase in twenty-four species of plant and animal. The thetin transmethylase activity of the green plants, moulds and samples of baker's yeast studied was negligible. Of the four invertebrates examined, only the common shore crab showed possible slight activity. The enzyme was present in appreciable amounts in the liver of all eleven vertebrates tested, and also in small amounts in rat and guinea-pig kidney. It was found to be absent from the spleen, brain and muscle of the rat, mouse and guinea pig.

It is a pleasure to thank Dr A. G. Hamilton and Dr R. R. Davies for much helpful advice and for the supply of some of the biological material used. Thanks are also due to Mr Norman Allin for his technical assistance in this work.

REFERENCES

Butz, L. W. & du Vigneaud, V. (1932). *J. biol. Chem.* **99**, 135.

Bywood, R. & Challenger, F. (1953). *Biochem. J.* **53**, xxvi.

Challenger, F. & Hayward, B. J. (1954). *Chem. & Ind.* p. 729.

Challenger, F. & Simpson, M. I. (1948). *J. chem. Soc.* p. 1591.

Dixon, M. & Webb, E. C. (1958). *Enzymes*, p. 246. London: Longmans, Green and Co.

Dubnoff, J. W. & Borsook, H. (1948*a*). *Fed. Proc.* **7**, 152.

Dubnoff, J. W. & Borsook, H. (1948*b*). *J. biol. Chem.* **176**, 789.

Durell, J. & Anderson, D. G. (1956). *Fed. Proc.* **15**, 245.

Durell, J., Anderson, D. G. & Cantoni, G. L. (1957). *Biochim. biophys. Acta*, **26**, 270.

Ericson, L.-E. (1958*a*). *Acta chem. scand.* **12**, 1541.

Ericson, L.-E. (1958*b*). *Abstr. 4th Int. Congr. Biochem., Vienna*, nos. 4–58, p. 45.

Kensler, C. J. & Langemann, H. (1954). *Proc. Soc. exp. Biol., N.Y.*, **85**, 364.

Krebs, H. A., Gurin, S. & Eggleston, L. V. (1952). *Biochem. J.* **51**, 614.

Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.

McRorie, R. A., Sutherland, G. L., Lewis, M. S., Burton, A. D., Glazener, M. R. & Shive, W. (1954). *J. Amer. chem. Soc.* **76**, 115.
Maw, G. A. (1955). *Biochem. J.* **60**, xxxii.
Maw, G. A. (1956). *Biochem. J.* **63**, 116.
Maw, G. A. (1958). *Biochem. J.* **70**, 168.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Riegel, B. & du Vigneaud, V. (1935–36). *J. biol. Chem.* **112**, 149.
Schlenk, F. & DePalma, R. E. (1956). *Fed. Proc.* **15**, 348.
Shapiro, S. K. (1958). *Biochim. biophys. Acta*, **29**, 405.
Sloane, N. H., Boggiano, E. M., Coulomb, B. & Hutchings, B. L. (1956). *Fed. Proc.* **15**, 357.
Thoai, N. v. & Robin, Y. (1956). *Bull. Soc. Chim. biol., Paris*, **38**, 843.

An Explanation for the Difference between the Responses of Mammals and Birds to Thyroxine and Tri-iodothyronine

By J. R. TATA* and C. J. SHELLABARGER†
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 28 *January* 1959)

It is now agreed that in various biological tests in mammals, and in amphibian metamorphosis, 3:5:3′-tri-iodo-L-thyronine is about two to six times as potent as L-thyroxine (Barker, 1955). This difference in potency in mammals is maintained over a wide variety of physiological potency tests involving basal metabolic rate, body growth, serum cholesterol and antigoitrogenic action. However, Shellabarger (1955) and Newcomer (1957) reported that, in the chicken, tri-iodothyronine had the same potency as thyroxine whatever the parameter used for comparison. This is the only example of a lack of difference in the potencies of these hormones when measured by all the above tests and no explanation was produced to account for it.

In most mammals the higher potency of tri-iodothyronine is accompanied by a more rapid action, which can be attributed to a faster rate of diffusion from blood to tissues than that of thyroxine. This concept is upheld by the two to four times shorter biological half-life of tri-iodothyronine (Berson, 1956) and the comparatively feeble affinity for serum thyroxine-binding proteins (Robbins & Rall, 1957). In order to determine whether the similar responses of the chicken to the two hormones were due to similar rates of diffusion into tissues, the biological half-lives of thyroxine and tri-iodothyronine in the chicken were first determined. A study was then made of thyroxine-binding protein in chicken and duck sera, with special regard to its relative affinity for the two hormones. It was assumed that the rate of disappearance of ^{131}I from the whole body gave a satisfactory indication of the biological half-lives of the ^{131}I-labelled hormones. The results show that both thyroid hormones have similar biological half-lives in the chicken; this can be accounted for by fundamental differences between the binding of thyroxine and tri-iodothyronine by chicken- and duck-serum proteins and by mammalian-serum proteins.

* Beit Memorial Fellow.

† Special Fellow, NIAMD, U.S. Public Health Service. Present address: Brookhaven National Laboratory, Upton, L.I., N.Y.

EXPERIMENTAL

Groups of five 21-day-old male chickens [Light Sussex × Rhode Island] were used for the determination of biological half-lives of L-thyroxine and 3:5:3′-tri-iodo-L-thyronine. Thyroxine (1 μg.) and tri-iodothyronine (0·82 μg.), labelled with ^{131}I with specific radioactivities of 22·1 mc and 24·3 mc/μmole respectively, were injected into each bird. The rates of disappearance of the two hormones were measured by counting the residual radioactivity in a total body ring γ-counter as described by Campbell, Cuthbertson, Matthews & McFarlane (1956), against suitable standards of each labelled compound. Measurements were made at time intervals of 5 min.–15 hr. up to 72 hr. after the intravenous injection of ^{131}I-labelled compounds. The results are expressed as the percentage of initial radioactivity found in the birds at time intervals studied.

The association of thyroid hormones with serum proteins was studied qualitatively by zone electrophoresis on paper according to methods described by Robbins & Rall (1957). Whatman no. 3 MM paper was used with 0·075 M-veronal (sodium barbiturate–barbituric acid) buffer, pH 8·6 (Block, Durrum & Zweig, 1955); radioactivity was localized by an automatically recording end-window β-counter and protein fractions distributed on paper were stained with bromophenol blue. The binding of endogenously labelled thyroid hormones to serum proteins was studied in the blood of 31-day-old chickens, 24 hr. after the administration of 0·8 mc of carrier-free [^{131}I]iodide per bird. Electrophoretic analysis of such serum samples was performed only after chromatographic methods had established that 90% of circulating organic ^{131}I in butanol extracts consisted of thyroxine and tri-iodothyronine. For binding studies *in*

vitro, electrophoresis was performed on serum obtained from 21- to 31-day-old chickens and White Peking ducks (1·5 kg.). Less than 0·01 μg. of ^{131}I-labelled L-thyroxine and L-tri-iodothyronine were added to 1 ml. of serum. Human serum, under identical conditions, was used throughout this work for comparison of thyroxine-binding properties. These studies were extended to the binding of thyroid hormones to isolated serum-protein fractions. For this purpose, chicken serum was fractionated in batches of 10 ml. by a method similar to that used for human-serum proteins (Lever *et al.* 1951). Each protein fraction was dissolved in 0·15 M-NaCl at a concentration which corresponded to that in whole serum. Serum proteins were also isolated by zone electrophoresis on cellulose columns according to methods described by Flodin & Porath (1954).

Quantitative comparisons of thyroxine-binding powers of whole human and avian sera and their protein fractions were made by a method based on a recently described property of thyroxine and related iodophenols (Tata, 1959*a*). The principle of the new method involves the 'stabilization' of thyroxine and tri-iodothyronine by thyroxine-binding proteins during the ionization of their phenolic hydroxyl groups. The degree of 'stabilization' or the inhibition of the spontaneous loss of thyroxine or tri-iodothyronine is a direct function of the fraction of hormone bound and the concentration of binding proteins (Tata, 1959*b*).

The actual procedure consisted of measuring on paper chromatograms the inhibition of loss of ^{131}I-labelled thyroxine or tri-iodothyronine (as percentage of total ^{131}I) in the first 5 min. after the mixing of 0·10–0·18 μg. of the hormone, dissolved in 0·04 ml. of aqueous 20% propane-1:2-diol, with 2·5 ml. of 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–maleate buffer (μ, 0·10; pH, 7·45). The inhibition of loss of radioactive material after its passage from propane-1:2-diol to the aqueous buffer was plotted against protein concentration when increasing amounts of avian or human serum or their protein fractions were added to the buffer. The protein concentrations required to produce binding or 'stabilization' of 50% of thyroxine were compared to obtain relative binding powers. At high protein concentrations there was agreement between the results obtained by this method and those obtained by the conventional electrophoretic method of displacement of bound thyroxine by saturation of the binding protein. The relative intensities with which the different thyroxine-binding components of human and avian sera bind the two hormones were measured by the electrophoretic method of displacement as described by Robbins & Rall (1957).

RESULTS

Average rates of loss of whole body radioactivity for ^{131}I-labelled L-thyroxine and 3:5:3′-tri-iodo-L-thyronine, injected into groups of five chickens, are shown in Fig. 1. The disappearance rates of the two labelled hormones were indistinguishable, a result which is different from that described for mammals. From data in Fig. 1, the biological half-life values ($T_{\frac{1}{2}}$) for thyroxine and tri-iodothyronine were calculated to be $22·5 \pm 1·0$ hr.

Since the difference in the relative binding affinities of the two compounds to serum proteins is reflected in the $T_{\frac{1}{2}}$ values for thyroxine and tri-iodothyronine in mammals, the next step was to compare thyroxine-binding by human serum and by chicken and duck sera. At first, qualitative experiments were performed in which the distribution of radioactivity was measured in human- and avian-serum proteins separated by paper electrophoresis. An example of such a comparison is presented in Fig. 2. A thyroxine-binding pattern, typical of that in most mammalian sera (Robbins & Rall, 1957), was observed for human serum; about 90% of added radioactive thyroxine migrated in a position intermediate between human α_1- and α_2-globulin (Fig. 2*A*). Less than 10% of ^{131}I was localized in human-serum albumin at low levels of added thyroxine. Under identical conditions, a different thyroxine-binding pattern was observed with chicken serum, whether radioactive thyroxine was added *in vitro* (Fig. 2*B*) or whether the distribution of endogenously labelled thyroid hormone was studied (Fig. 2*C*). In the chicken serum, over 80% of the total ^{131}I was localized in the albumin fraction, whereas only about 10% of the radioactivity could be detected in the α-globulin fraction. A qualitatively similar pattern was obtained with duck serum; further, chicken- and duck-serum albumins are also the principal binders of tri-iodothyronine when it is added in trace amounts to both chicken and duck sera.

Myant (1957) has shown that the thyroxine-binding proteins in rat serum cannot be distinguished from albumin under certain conditions of

Fig. 1. Whole body disappearance rates of ^{131}I-labelled L-thyroxine and 3:5:3′-tri-iodo-L-thyronine in the chicken. Radioactive thyroxine (1 μg.) and tri-iodothyronine (0·82 μg.) were injected intravenously into groups of five birds. The residual radioactivity was counted in a total body ring γ-counter. ○, Thyroxine; △, tri-iodothyronine.

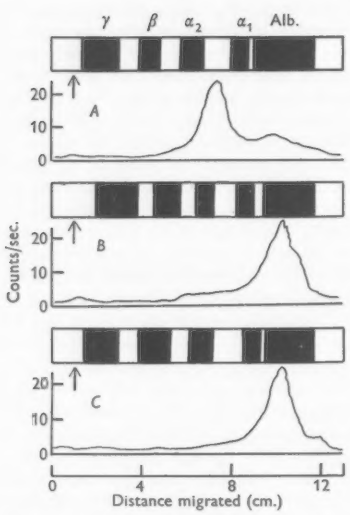


Fig. 2. Thyroxine-binding in human and chicken sera as determined by paper-electrophoretic analysis in 75 mM-veronal (sodium barbiturate–barbituric acid) buffer, pH 8·8, at 4 v/cm. for 18 hr. The bands on top show the distribution of γ-, β-, α_1- and α_2-globulin and albumin (Alb.); below them is shown the distribution of radioactivity after ^{131}I-labelled thyroxine was added to the serum or if endogenously labelled hormone was present. Vertical arrows indicate the points of application of samples. *A*, Human serum + ^{131}I-labelled L-thyroxine (0·08 μg./ml.); *B*, chicken serum + radioactive L-thyroxine (0·07 μg./ml.); *C*, chicken serum obtained from a bird 24 hr. after the injection of carrier-free [^{131}I]iodide.

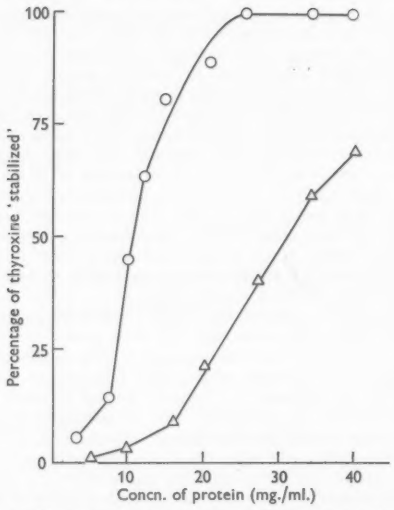


Fig. 3. Thyroxine-binding power of human and chicken sera at different dilutions. Thyroxine-binding is expressed as a percentage of thyroxine 'stabilized' at a given serum-protein concentration. 'Stabilization' was measured as follows: 0·015 μg. of ^{131}I-labelled L-thyroxine dissolved in 20% 1:2-propanediol was mixed with different amounts of human or chicken serum diluted in 0·05M-tris–maleate buffer, pH 7·45. The percentage of inhibition (as compared with a protein-free control) of the disappearance of radioactivity in the thyroxine fraction at 5 min. after mixing in the presence of different amounts of serum was determined chromatographically. Percentage of inhibition of disappearance = percentage of thyroxine 'stabilized' or protein-bound (see Tata, 1959*b*). ○, Human serum; △, chicken serum.

Table 1. *Binding of* ^{131}I-*labelled* L-*thyroxine to protein fractions isolated from human and chicken sera*

The final concentration of added radioactive thyroxine was 63 μμM. The percentage of protein-bound ^{131}I was determined by paper electrophoresis in 0·075M-veronal (sodium barbiturate–barbituric acid) buffer, pH 8·6.

Species	Protein fraction	Method of isolation	Concn. of protein (mg./ml.)	Percentage of ^{131}I bound to protein
Man	γ-Globulin	Ethanol fractionation	7·5	12·0–18·0*
	β-Globulin	Electrophoresis	5·0	6·0
	α_1- + α_2-Globulin	Ethanol fractionation	5·0	96·5
		Electrophoresis	5·0	100·0
	Albumin	Ethanol fractionation	15·0	42·0
		Electrophoresis	15·0	64·6
Chicken	γ-Globulin	Ethanol fractionation	7·5	8·5–15·5*
	β-Globulin	Electrophoresis	7·5	9·0
	α_1- + α_2-Globulin	Ethanol fractionation	7·5	20·8
		Electrophoresis	7·5	14·3
	Albumin	Ethanol fractionation	15·0	35·5
		Electrophoresis	15·0	52·1

* It was difficult to obtain accurate values because of the low electrophoretic mobility of γ-globulin.

Table 2. *Comparison of affinity and capacity of human and chicken sera to bind thyroxine*

Serum samples were diluted with 0·15 M-NaCl and 'stabilization' was measured in 0·05 M-tris–maleate buffer, pH 7·5, at 37° (Tata, 1959*b*).

Species	Serum dilution	Concn. of thyroxine (μg./ml.) 0·05	0·10	0·20	0·40
		Percentage of thyroxine stabilized			
Man	None	99·0	100·0	96·4	85·5
	1:2	98·0	100·0	91·2	75·0
	1:4	92·0	83·7	80·3	63·3
	1:8	57·6	47·1	28·4	23·1
	1:16	10·1	5·3	<5·0	<2·0
Chicken	None	100·0	94·5	78·2	42·2
	1:2	78·1	56·0	38·0	24·8
	1:4	28·0	22·6	10·5	8·3
	1:8	5·2	<5·0	<2·0	<2·0
	1:16	0	0	<2·0	0

Table 3. *Relative binding affinities of the major thyroxine-binding components of human, chicken and duck sera for* 3:5:3′*-tri-iodothyronine*

The values are expressed relative to the L-thyroxine-binding affinity of the particular species, taken to be 100 in each case.

Species	Sample	Concn. of protein (mg./ml.)	Relative binding affinity for tri-iodothyronine
Man	Whole serum	—	34
	Cohn fraction IV-4	7·5	30
	Prealbumin	2·0	25
	Albumin	25·0	84
Chicken	Whole serum	—	84
	Albumin	20·0	92
Duck	Whole serum	—	79
	Albumin	20·0	87

electrophoresis. In order to test further for the absence in avian serum of a thyroxine-binding protein comparable with the human protein, radioactive thyroxine was added to individual human- and chicken-serum-protein fractions after their isolation by fractionation with ethanol or by electrophoresis. As demonstrated in Table 1, chicken-serum albumin was still found to be the principal thyroxine-binding protein, whereas in human-serum proteins isolated by identical procedures the α-globulin-rich fractions were the principal thyroxine-binding fractions. Substantial binding was also exhibited by isolated human-serum albumin.

These differences in thyroxine-binding patterns were further demonstrated by the following quantitative studies: (1) a comparison of the thyroxine-binding powers of whole human and avian sera; (2) estimation of the relative affinities of the principal thyroxine-binding components of human, chicken and duck sera for thyroxine and tri-iodothyronine. Thyroxine-binding powers of whole sera were compared by measuring the fraction of thyroxine (present in a very low concentration) 'stabilized' or protein-bound in the presence of increasing dilutions of serum; this is illustrated in Fig. 3. From these results, it follows that human serum exhibits a higher binding power than chicken or duck sera. For example, if the protein concentration necessary to bind 50% of the thyroxine is used as an arbitrary standard of comparison in data presented in Fig. 3, less human serum (10 mg. of protein/ml.) is required than chicken or duck serum (30 and 25 mg. of protein/ml. respectively) to obtain the same result. Alternatively, if the serum dilutions at which ability to bind thyroxine is lost are compared, it will be seen that for chicken and duck sera a four- to five-fold dilution was effective whereas human serum had to be diluted to one-tenth of its normal concentration. The curves in Fig. 3 cannot by themselves distinguish between 'affinity' and 'capacity' to bind thyroxine; for this purpose similar measurements were made in the presence of increasing concentrations of thyroxine (Table 2). When data from Fig. 3 and Table 2 are combined it is evident that chicken serum possesses both a lower affinity and capacity to bind thyroxine than human serum.

Comparison of the relative intensities with which human, chicken and duck sera and their constituent proteins bind thyroxine and tri-iodothyronine

revealed a further difference in thyroxine-binding in these species (Table 3). Whole human serum and its major thyroxine-binding protein components, Cohn fraction IV-4 (Freinkel, Dowling & Ingbar, 1955) and prealbumin (Schultze, Schönenberger & Schwick, 1956), all bind thyroxine about three to four times more firmly than tri-iodothyronine. In contrast, human-serum albumin, which is a secondary binding protein (i.e. the binding is evident only at thyroxine concentrations above 0·2 μM), exhibits little difference in the avidity with which it binds either hormone. Under identical conditions, whole chicken and duck sera and their serum-albumin fraction (which is their only thyroxine-binding protein component) bound thyroxine and tri-iodothyronine with nearly the same affinity. These last observations indicate the absence from avian blood of a thyroxine-binding protein capable of binding thyroxine more firmly than tri-iodothyronine.

DISCUSSION

The difference between the relative biological potencies of thyroxine and tri-iodothyronine in mammals and birds are also manifest in their disappearance rates. The identity in the biological half-lives of thyroxine and tri-iodothyronine in the chicken is the first example of its kind; in several mammalian species such as man (Sterling, Lashof & Man, 1954; Ingbar & Freinkel, 1955) and rat (Maclagan & Wilkinson, 1954; Feldman, 1957), tri-iodothyronine disappears from the blood as well as from the whole body about two to three times more rapidly than does thyroxine. The low figure of $T_{\frac{1}{2}}$ (22·5 $\pm$ 1 hr.) for the two hormones in the chicken is closer to the value for tri-iodothyronine than for thyroxine in a mammalian species of comparable body weight. The short biological half-life of thyroid hormones in the chicken may explain the low protein-bound iodine values in chicken serum reported by Mellen & Hardy (1957).

The present studies on the transport of thyroid hormones in blood show that the above-mentioned differences between mammals and birds also hold for their interaction with serum proteins. In a variety of mammals, as well as in reptiles (Robbins & Rall, 1957) and fish (Tata, unpublished work), thyroxine and tri-iodothyronine are bound in the blood to a thyroxine-binding protein fraction which is characterized by a mobility in paper electrophoresis intermediate between that of α_1- and α_2-globulin. This specific protein fraction is absent from, or barely discernible in, chicken and duck sera (Fig. 2 and Table 1). The study of thyroxine-binding by protein fractions isolated from human, chicken and duck sera by fractionation with ethanol further demonstrates this lack of thyroxine-binding protein in avian serum. Ingbar, Dowling & Freinkel (1957) showed that the human α-globulin-rich fractions, isolated by similar procedures (Cohn fractionation), constitued the most active thyroxine-binding protein fraction. Hence there exists a fundamental difference in the pattern in which thyroid hormones are distributed and bound to mammalian- and avian-serum proteins.

To give meaning to this qualitative difference in the nature of thyroxine-binding, a definition in terms of 'affinity' and 'capacity' for thyroxine-binding was necessary for a correct physiological interpretation. It is here that a useful application of a newly discovered property of thyroxine and the effect of proteins on it (Tata, 1959*a*, *b*) has been made. The data presented in Fig. 3 and Table 2 in terms of 'stabilization' of thyroxine as a measure of thyroxine-binding are interpreted to mean that the difference in binding pattern is accompanied by a lower affinity and capacity of chicken serum to interact with thyroxine than that of human serum. Further, human serum and sera of other mammalian species exhibit a markedly greater affinity for thyroxine than for tri-iodothyronine, whereas in chicken or duck serum both hormones are bound with about the same intensity. Earlier work had similarly shown the higher avidity of human serum for thyroxine (Robbins & Rall, 1955; Larson & Albright, 1955), and results presented in Table 3 further show that a selectively firmer binding of thyroxine is confined to the major thyroxine-binding protein fractions in human serum; the α-globulin-rich fraction or thyroxine-binding protein and the recently isolated prealbumin (Schultze *et al.* 1956). However, the same results also show that thyroxine-binding proteins, whether human or avian, can be divided into two classes: (1) those that bind thyroxine more firmly than tri-iodothyronine, and (2) those that bind the hormones with equal avidity. The second class consists of both human- and avian-serum albumins, indicating a common property of serum albumins irrespective of species. The principal difference between mammalian and avian sera is a lack of a specific thyroxine-binding protein in the latter.

The different metabolic and other responses exhibited by mammals and birds to thyroxine and tri-iodothyronine appear to result from the relative speeds of diffusion of these compounds from blood to tissue cells. The very similar disappearance rates of the two hormones in the chicken and the fact that they are equally strongly bound to avian-serum proteins are undoubtedly related, and the shorter biological half-life of tri-iodothyronine in mammals can similarly be accounted for by the loose nature of its binding to serum proteins compared with that of thyroxine. This supports the hypothesis that the biological potency of the

thyroid hormones in a given animal is determined by the rapidity with which they leave the blood and reach their intracellular sites of action. The study of thyroid-hormone transport in birds has therefore been valuable in revealing the physiological significance of similar data previously obtained in mammals.

SUMMARY

1. The similar potencies of L-thyroxine and 3:5:3′-tri-iodo-L-thyronine in the chicken have been further studied by comparing the overall rate of metabolism of the two thyroid hormones in both mammals and birds and by comparing binding of thyroxine by serum proteins in man, chicken and duck.

2. Thyroxine and tri-iodothyronine have indistinguishable rates of disappearance from the whole body in the chicken with half-lives of $22{\cdot}5 \pm 1$ hr.

3. Thyroxine-binding protein as found in human serum is absent from, or present only in small amounts in, chicken and duck sera. Both endogenously labelled and exogenously labelled thyroid hormones are mostly bound to albumin in the sera of these birds.

4. Quantitative studies show that both affinity and capacity of chicken serum to bind thyroxine are lower than those found for human serum. The major thyroxine-binding protein fractions in human serum, Cohn fraction IV-4 and prealbumin, bind thyroxine about three to four times more firmly than tri-iodothyronine. Human-, chicken- and duck-serum-albumin fractions bind both hormones with almost identical intensities.

5. It is concluded that the difference in the response of birds and mammals to tri-iodothyronine, as compared with thyroxine, is due to a fundamental difference in the binding of thyroid hormones to avian-serum proteins and mammalian-serum proteins respectively.

We would like to thank Dr R. Pitt-Rivers, F.R.S., for helpful suggestions, Professor H. E. Schultze for the sample of human-serum prealbumin and Dr S. H. Ingbar for Cohn fraction IV-4.

REFERENCES

Barker, S. B. (1955). *Annu. Rev. Physiol.* **17**, 417.
Berson, S. A. (1956). *Amer. J. Physiol.* **20**, 653.
Block, R. J., Durrum, E. L. & Zweig, G. (1955). *A Manual of Paper Chromatography and Electrophoresis.* New York: Academic Press Inc.
Campbell, R. M., Cuthbertson, D. P., Matthews, C. M. & McFarlane, A. S. (1956). *Int. J. appl. Radiat. Isotopes*, **1**, 66.
Feldman, J. D. (1957). *Amer. J. Physiol.* **188**, 30.
Flodin, P. & Porath, J. (1954). *Biochim. biophys. Acta*, **28**, 91.
Freinkel, N., Dowling, J. T. & Ingbar, S. H. (1955). *J. clin. Invest.* **34**, 1698.
Ingbar, S. H., Dowling, J. T. & Freinkel, N. (1957). *Endocrinology*, **61**, 321.
Ingbar, S. H. & Freinkel, N. (1955). *J. clin. Invest.* **34**, 1375.
Larson, F. C. & Albright, E. C. (1955). *Endocrinology*, **56**, 737.
Lever, W., Gurd, F. R. N., Uroma, E., Brown, R. K., Barnes, B. A., Schmid, K. & Schultz, E. L. (1951). *J. clin. Invest.* **30**, 99.
Maclagan, N. F. & Wilkinson, J. H. (1954). *J. Physiol.* **125**, 405.
Mellen, W. J. & Hardy, L. B. jun. (1957). *Endocrinology*, **60**, 547.
Myant, N. B. (1957). *J. Physiol.* **135**, 426.
Newcomer, W. S. (1957). *Amer. J. Physiol.* **190**, 413.
Robbins, J. & Rall, J. E. (1955). *J. clin. Invest.* **34**, 1331.
Robbins, J. & Rall, J. E. (1957). *Recent Progr. Hormone Res.* **13**, 161.
Schultze, H. E., Schönenberger, M. & Schwick, G. (1956). *Biochem. Z.* **328**, 267.
Shellabarger, C. J. (1955). *Poultry Sci.* **34**, 1437.
Sterling, K., Lashof, J. C. & Man, E. B. (1954). *J. clin. Invest.* **33**, 1031.
Tata, J. R. (1959*a*). *Biochem. J.* **72**, 214.
Tata, J. R. (1959*b*). *Biochem. J.* **72**, 222.

Inhibition of Aconitase by Glyoxylate plus Oxaloacetate

By A. RUFFO, MARTA ROMANO and ANNA ADINOLFI
Laboratory of Biochemistry, Institute of Tumours, 'Pascale Foundation', Naples, Italy

(*Received* 18 *December* 1958)

Previous experiments showed that glyoxylate incubated with oxaloacetate in homogenates of rat liver and other animal tissue (D'Abramo, Romano & Ruffo, 1957*a*, *b*; 1958) produced a complete inhibition of citrate oxidation. The inhibition was explained by supposing that the two substances might react together to form a compound of six carbon atoms, possibly oxalomalic acid, which, even at very low concentration, could inhibit citrate oxidation by competition with aconitase or *iso*-citric dehydrogenase. Of these, aconitase was the first enzyme tested for the possibility that it could be inhibited by a condensation compound formed during the incubation of glyoxylate with oxaloacetate.

The results of these experiments show that a complete inhibition of citrate formation from *cis*-aconitate occurred in both crude and purified

aconitase preparations, when the incubation was in the presence of oxaloacetate plus glyoxylate. Evidence is also presented that oxaloacetate and glyoxylate react together non-enzymically in the presence of magnesium to produce an inhibitor of aconitase.

EXPERIMENTAL

Preparation of crude aconitase. The method of Johnson (1939) was followed, rat liver being used instead of pigeon-breast muscle. The liver was cut into small pieces, ground in a mortar at 0° and mixed with 5 vol. of 0·1 M-phosphate buffer (17·8 g. of $Na_2HPO_4,2H_2O$ and 20 ml. of N-HCl to 1000 ml. of water); pH was 7·4. The mixture was centrifuged at 0° (4000 rev./min. for 10 min.) and the supernatant was diluted (1:10) with 0·1 M-phosphate buffer containing 0·9% of KCl. A sample (3 ml.) of the clear extract was used for the activity determinations.

Purification of aconitase from pig heart. The method of Anfinsen (1955), based on ethanol fractionation of pig-heart homogenate, followed by fractionation with ammonium sulphate, was used. An 18-fold-purified preparation was obtained in the form of a pale-pink amorphous precipitate, which was dissolved before use in 0·1 M-phosphate buffer (as described above) containing 0·05 M-sodium citrate; pH was 8·2. These solutions contained 2–3 mg. of protein/ml. and approx. 50 units/mg. Portions (0·1–0·2 ml.) of these solutions were used for the single determinations.

Determination of aconitase activity. In crude extracts the activity was determined by incubating 10 μmoles of *cis*-aconitate (Sigma Chemical Co., St. Louis, Mo., U.S.A.) with 3 ml. of diluted rat-liver extract and adjusting the volume to 4 ml. with the phosphate buffer–KCl. The time of incubation at 38° was 1 hr., unless otherwise indicated, and the reaction was stopped by adding 3 ml. of freshly prepared 5% (w/v) tungstic acid.

With the purified preparations the assay method suggested by Anfinsen (1955) was followed. A volume (0·5 ml) of 0·2 M-*cis*-aconitate solution (100 μmoles) was incubated with 0·1–0·2 ml. of enzyme diluted to a final volume of 4 ml. with 0·1 M-phosphate buffer, pH 7·4. The incubation, carried out at 38° for 30 min., was stopped by adding 0·5 ml. of 30% (w/v) trichloroacetic acid.

Citrate determination. Citrate was determined in the tungstic acid filtrates by the method of Ettinger, Goldbaum & Smith (1952), which was most suitable for the determination of small amounts of citrate, and in the trichloroacetic acid filtrates by the method described by Stern (1957).

Where indicated, the enzyme preparations were incubated with glyoxylate or oxaloacetate (L. Light and Co. Ltd., Colnbrook, Bucks) or both. The concentrations used do not interfere in the determination of citrate.

Absorption spectra and chromatographic determination of 2:4-dinitrophenylhydrazones. Two portions (0·1 and 0·4 ml.) of the trichloroacetic acid filtrates, after dilution to 2 ml. with water, were treated with 0·5 ml. of 0·2% (w/v) 2:4-dinitrophenylhydrazine. To the first portion, 2·5 ml. of 2 N-NaOH was added for the determination of absorption spectra. The readings were done with a Beckman spectrophotometer between 400 and 600 mμ, against a blank of the phenylhydrazine, water and NaOH soln. The second was treated with formaldehyde according to Cavallini & Mondovì (1957), extracted with ether, dried *in vacuo* and redissolved in 0·8 ml. of aq. N-NH_3 soln.–$CHCl_3$ (1:1, v/v). A portion (0·05 ml.) of the aqueous phase was spotted on Schleicher und Schüll (Dassel, Germany) no. N. 2043*b* filter paper, and chromatographed, by descending chromatography, for 14 hr. in butan-1-ol saturated with aq. 3% (v/v) NH_3 soln. The spots were developed with 2 N-NaOH. The results of these experiments are reported in Table 3. Fig. 3 shows the chromatogram of the phenylhydrazones obtained from the filtrates of the experiment with purified aconitase (Table 2, Expt. 3).

RESULTS

Inhibition of crude aconitase. Rat-liver extracts diluted in 0·1 M-phosphate buffer, pH 7·4, containing 0·9% of KCl were incubated in a final volume of 4 ml. with 10 μmoles of *cis*-aconitate in the presence and in the absence of different amounts of glyoxylate and oxaloacetate, added alone and together, as reported in Table 1.

The results showed that whereas the addition of either oxaloacetate or glyoxylate alone did not produce any effect on the formation of citrate from

Table 1. *Inhibition of crude aconitase by glyoxylate plus oxaloacetate*

A portion (3 ml.) of rat-liver extract diluted (1:50) with phosphate buffer–KCl, pH 7·4, was used. Additions: *cis*-aconitate (10 μmoles), glyoxylate and oxaloacetate as indicated. Final volume, 4 ml.; incubation, 60 min.; temp. 38°. For deproteinization 3 ml. of 5% (w/v) tungstic acid was used.

Expt.	Additions	Citrate found (μmoles)	Inhibition (%)
1	*cis*-Aconitate	7·7	—
	cis-Aconitate + 4 μmoles of glyoxylate	7·7	0
	cis-Aconitate + 8 μmoles of oxaloacetate	7·7	0
	cis-Aconitate + oxaloacetate + glyoxylate	0·4	94
2	*cis*-Aconitate	8·4	—
	cis-Aconitate + 5 μmoles of glyoxylate	8·2	2
	cis-Aconitate + 5 μmoles of oxaloacetate	8·1	3
	cis-Aconitate + glyoxylate + oxaloacetate	0	100
3	*cis*-Aconitate	7·1	—
	cis-Aconitate + 2 μmoles of glyoxylate	7·0	1
	cis-Aconitate + 2 μmoles of oxaloacetate	7·0	1
	cis-Aconitate + oxaloacetate + glyoxylate	0·9	87

cis-aconitate, when these substances were added together almost a complete inhibition of the reaction occurred. Since the inhibition was 87 % also with amounts of glyoxylate and oxaloacetate as low as 2 μmoles/flask (0·5 mM), the effect of lower concentrations of glyoxylate and of oxaloacetate was investigated. The results in Fig. 1 show that approximately 50 % inhibition was obtained for the addition of very low amounts (0·1 μmole; 25 μM) of either oxaloacetate (to the incubation mixtures containing glyoxylate) or glyoxylate (to the incubation mixtures containing oxaloacetate). The inhibition reached maximal values with amounts of either glyoxylate or oxaloacetate between 0·4 and 0·6 μmole/flask (0·1–0·15 mM).

Another point investigated was the effect of the time of incubation on the rate of inhibition. The curves of Fig. 2 (*a*) show that in the presence of glyoxylate and oxaloacetate the formation of citrate from *cis*-aconitate was inhibited at the beginning of the incubation; those of Fig. 2 (*b*) show that between only 4 and 6 min. of incubation the inhibition was already evident.

Inhibition of purified aconitase. The results reported in Table 2 were obtained with a purified aconitase preparation from pig heart according to the method of Anfinsen (1955). They show that

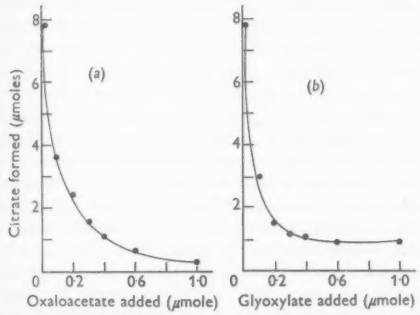


Fig. 1. Inhibition of aconitase by oxaloacetate and glyoxylate. (*a*) Incubation mixtures contained 4 μmoles of glyoxylate and oxaloacetate as indicated. (*b*) Incubation mixtures contained 4 μmoles of oxaloacetate and glyoxylate as indicated. With both (*a*) and (*b*) incubation was for 1 hr. at 38°, with 10 μmoles of *cis*-aconitate and 3 ml. of diluted (1:50) liver extract in 0·1 M-phosphate buffer, pH 7·4, containing 0·9 % of KCl. Final volume, 4 ml. For deproteinization 3 ml. of freshly prepared 5 % (w/v) tungstic acid was used.

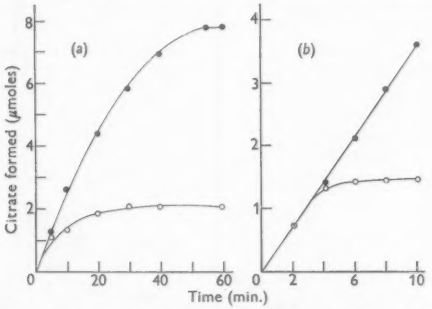


Fig. 2. Rate of inhibition of aconitase. ●, Incubation mixtures contained 3 ml. of diluted (1:50) liver extract in 0·1 M-phosphate buffer containing 0·9 % of KCl, and 10 μmoles of *cis*-aconitate; ○, incubation mixtures, contained in addition 4 μmoles each of oxaloacetate and glyoxylate. Final volumes, 4 ml. The reaction was stopped by the addition of 3 ml. of 5 % (w/v) tungstic acid at the times indicated. (*a*) Incubation was at 38° for 1 hr.; (*b*) incubation was at 38° for 10 min.

Table 2. *Inhibition of purified aconitase by glyoxylate plus oxaloacetate*

Enzyme was present as indicated. Additions: *cis*-aconitate, 100 μmoles; oxaloacetate, 5 μmoles if present; glyoxylate as indicated; 0·1 M-phosphate buffer, pH 7·4. Incubation was for 30 min. at 38°; final volume, 4 ml. For deproteinization 0·5 ml. of 30 % (w/v) trichloroacetic acid was used.

Expt.	Enzyme: Vol. (ml.)	Enzyme: Concn. (mg. of protein/ml.)	Additions	Citrate formed (μmoles)	Inhibition (%)
1	0·2	3·1	Nil	42	—
			Oxaloacetate	41	2
			Glyoxylate (50 μmoles)	40	4
			Oxaloacetate + glyoxylate	2	95
2	0·1	2·0	Nil	35	—
			Oxaloacetate	35	0
			Glyoxylate (5 μmoles)	35	0
			Oxaloacetate + glyoxylate	0	100
3	0·1	3·0	Nil	28	—
			Oxaloacetate	27	3
			Glyoxylate (5 μmoles)	28	0
			Oxaloacetate + glyoxylate	0	100

with this preparation the addition of small amounts (5 μmoles; 1·25 mM) of glyoxylate plus oxaloacetate produced almost a complete inhibition of citrate formation from *cis*-aconitate.

To investigate the mechanism of the inhibition, portions of the trichloroacetic acid filtrates of the last experiment (Table 2, Expt. 3) were taken for chromatographic assay of the phenylhydrazones. Results presented in Fig. 3 show the spots obtained from the different filtrates after incubation for 60 min. No phenylhydrazone spots appeared in sample no. 1, corresponding to the incubation of the enzyme with *cis*-aconitate, whereas the characteristic spots of glyoxylate and oxaloacetate were shown in nos. 2 and 3. A new spot, not far from the starting point, whose R_F was different from all the others, appeared in the filtrate from Expt. 4, where oxaloacetate and glyoxylate were incubated together and produced the inhibition of aconitase. The position of this new spot on the chromatogram was similar to that indicated by Cavallini & Frontali (1954) for the phenylhydrazone of oxalosuccinic acid (R_F approx. 0·07).

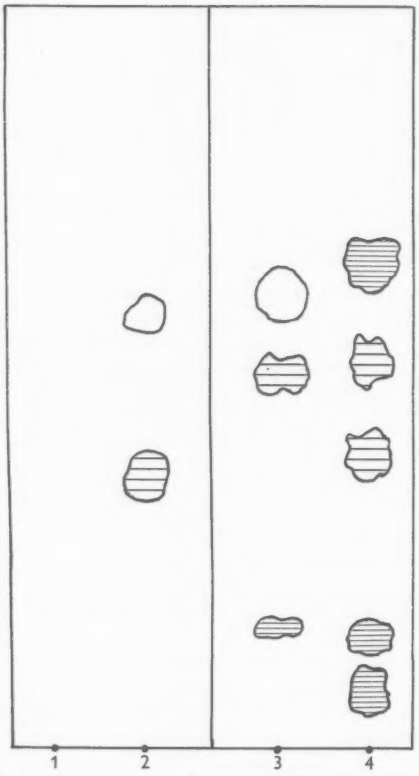


Fig. 3. Chromatogram of the phenylhydrazones obtained after incubation of oxaloacetate and glyoxylate with purified aconitase. 1, Enzyme only; 2, enzyme plus glyoxylate; 3, enzyme plus oxaloacetate; 4, enzyme plus oxaloacetate and glyoxylate. Incubation mixtures contained: 0·1 ml. of enzyme solution; 5 μmoles each of oxaloacetate and glyoxylate in 0·1 M-phosphate buffer, pH 7·4, to make the volume 4 ml. Incubation was at 38° for 30 min.; for deproteinization 0·5 ml. of trichloroacetic acid was used.

Incubation of glyoxylate plus oxaloacetate without the enzyme. The preceding results show that during the incubation of a mixture containing *cis*-aconitate, oxaloacetate, glyoxylate and aconitase, a new substance forming a phenylhydrazone appeared. It is possible that this substance formed from the reaction of oxaloacetate plus glyoxylate is responsible for the inhibition of aconitase. Since this reaction appeared to occur in the purified aconitase preparation without condensing enzymes, the possibility that this compound may be formed by a spontaneous (not enzymic) reaction of oxaloacetate with glyoxylate was investigated. Glyoxylate and oxaloacetate were incubated in Krebs phosphate saline free from Ca^{2+} ions (Umbreit, Burris & Stauffer, 1951), water, or 0·1 M-phosphate buffer, both in the presence and the absence of Mg^{2+} ions. In other experiments Mn^{2+} ions were substituted for Mg^{2+} ions. The chromatographic results (Table 3) showed that either oxaloacetate or glyoxylate after incubation for 1 hr. produced, usually, two spots of rather constant R_F. The spot with the lowest R_F (0·14–0·16) is the hydrazone of oxaloacetate, the second and the third are the hydrazones of pyruvate. This keto acid can give two spots, the *syn*- and *anti*-forms (Cavallini & Frontali, 1954), and this may account for our finding in the experiment with enzyme, where probably more oxaloacetate was converted into pyruvate (Fig. 3 and Table 3, Expt. 1). The R_F values of glyoxylate hydrazones were reasonably similar in the different media investigated, and comparable with the results of Cavallini & Frontali (1954), who also found two spots.

In the experiments in which oxaloacetate and glyoxylate were incubated together, a new hydrazone was formed whose R_F was different from those of either oxaloacetate or glyoxylate. This new substance appeared in the experiment with enzyme (Table 3, Expt. 1) and also in experiments where the incubation was carried out in Krebs phosphate saline (Expt. 2), in water (Expt. 4) and in phosphate buffer (Expt. 7), in which Mg^{2+} ions were present. It was not present in the media without Mg^{2+} ions (Expts. 3, 5 and 6). It should be noted that the position of this new spot on the chromatograms was near to the starting line (R_F 0·07), and appeared to be similar to that attributed by

Table 3. *Chromatographic separation of the phenylhydrazones of glyoxylate and oxaloacetate*

Glyoxylate (5 μmoles) or oxaloacetate (5 μmoles) or both were incubated at 38° for 1 hr. in 4 ml. of the media indicated below. A volume (0·5 ml.) of 30% (w/v) trichloroacetic acid was then added and after treatment with 2:4-dinitrophenylhydrazine portions corresponding to approx. 0·05 μmole of the original keto acids were spotted on papers. Results given are the R_F values of the single spots developed with 2N-NaOH In Expts. 6 and 7 portions from mixtures in which oxaloacetate or glyoxylate was the sole substrate have not been chromatographed.

		Substrates incubated		
Expt.	Incubation media	Glyoxylate	Oxaloacetate	Oxaloacetate plus glyoxylate
1	0·1M-Phosphate buffer, pH 7·4, and enzyme	0·36, 0·58	0·15, 0·58, 0·61	0·06, 0·14, 0·40 0·51, 0·64
2	Krebs phosphate saline (approx. 14 μg.atoms of Mg^{2+} ions)	0·38, 0·60	0·16, 0·57	0·07, 0·17, 0·40 0·57, 0·66
3	Water	0·31, 0·52	0·14, 0·42	— 0·14, 0·31 0·38, 0·50
4	Water plus 100 μg.atoms of Mg^{2+} ions	0·31, 0·52	0·14, —	0·05, 0·12, 0·30 — 0·52
5	Water plus 100 μg.atoms of Mn^{2+} ions	0·32, 0·55	0·14, 0·43	— 0·14, 0·32 0·35, 0·53
6	0·1M-Phosphate buffer, pH 7·4	— —	— —	— 0·15, 0·47 0·54, 0·66
7	0·1M-Phosphate buffer plus 100 μg.atoms of Mg^{2+} ions	— —	— —	0·07, 0·16, 0·44 0·53, 0·64

Cavallini & Frontali (1954) to oxalosuccinic acid, a tricarboxylic acid with six carbon atoms.

Moreover, the absorption spectra of the dinitrophenylhydrazones, obtained after similar incubation experiments, showed (Fig. 4) that a fall of absorption at 450 mμ occurred in the samples corresponding to the incubation of oxaloacetate plus glyoxylate in water and in the presence of Mg^{2+} ions (Fig. 4*d*). These results were not obtained in the absence of Mg^{2+} ions (Fig. 4*b*). Comparison of the curves (*b*) and (*d*) with the results reported in Table 3 shows that the fall of the absorption spectra, after the incubation of oxaloacetate plus glyoxylate, occurred only in the same experimental conditions in which the additional spot was detectable, i.e. in the presence of Mg^{2+} ions. Similar results, not reported in the text, were obtained also by incubating oxaloacetate plus glyoxylate in Krebs phosphate saline, and in phosphate buffer. The fall in absorption was observed only in the Krebs phosphate saline, which contains Mg^{2+} ions.

Effect of Mg^{2+} *ions on the inhibition of aconitase.* To confirm that the additional chromatographic spot appearing in the preceding experiments, when oxaloacetate and glyoxylate were incubated together in the presence of Mg^{2+} ions, was related to the formation of the inhibitor, crude aconitase was incubated in phosphate buffer in the absence of Mg^{2+} ions, with the concentration of oxaloacetate reduced to 0·06 μmole/flask to decrease the inhibition approximately to 50%. To similar incubation mixtures, increasing concentrations of Mg^{2+} ions were added, as indicated in Fig. 5, and citrate was determined after incubation for 1 hr.

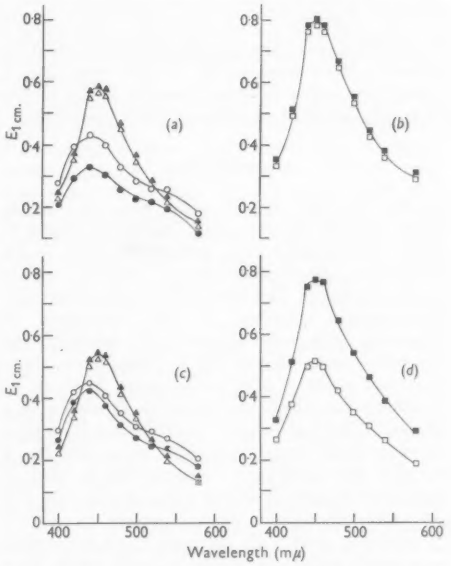


Fig. 4. Absorption curves of the phenylhydrazones obtained before (filled-in points) and after (unfilled points) incubation with 5 μmoles of oxaloacetate (○) or of glyoxylate (△) or glyoxylate plus oxaloacetate (□). Final volume, 4 ml.; incubation was at 38° for 1 hr. A volume (0·5 ml.) of 30% (w/v) trichloroacetic acid was added at the end of the incubation. (*a*) and (*b*) Incubation in water; (*c*) and (*d*) incubation in water plus 100 μg.-atoms of Mg^{2+} ions.

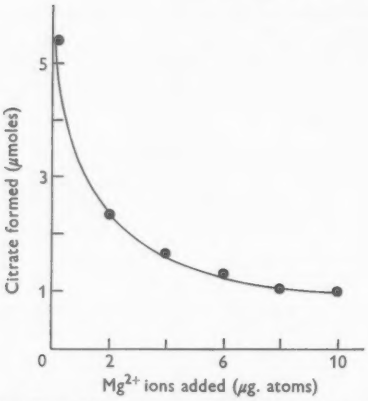


Fig. 5. Effect of Mg^{2+} ions on the inhibition of aconitase. The incubation mixture contained 3 ml. of diluted (1:50) liver extract in 0·1 M-phosphate buffer containing 0·9% of KCl, 0·06 μmole of oxaloacetate, 4 μmoles of glyoxylate and $MgCl_2$ as indicated, in the final volume of 4 ml. Incubation was at 38° for 1 hr.; for deproteinization 3 ml. of freshly prepared 5% (w/v) tungstic acid was used.

The results showed that the inhibition increased by increasing the concentration of Mg^{2+} ions until it reached the value of approximately 90%, at the concentration of 10 μg.atoms of Mg^{2+} ions/flask.

DISCUSSION

Results presented in this paper seem to confirm earlier ones (D'Abramo *et al.* 1958), according to which oxaloacetate and glyoxylate may react together to form a compound which inhibits citrate oxidation. The results show that aconitase was strongly inhibited by the incubation of glyoxylate plus oxaloacetate. Its activity was reduced between 75 and 100% irrespective of the degree of purity. Chromatographic analysis of the incubation mixtures showed the formation of a substance forming a hydrazone different from that of oxaloacetate and glyoxylate. Since the R_F of this new spot appeared to be similar to that of oxalosuccinic acid, it seems possible that the new spot observed in our chromatograms is another tricarboxylic acid with six carbon atoms, oxalomalic acid, which theoretically could result from the reaction of glyoxylate plus oxaloacetate. Evidence has been obtained that oxaloacetate and glyoxylate may react together without enzyme in media containing Mg^{2+} ions to produce an inhibitor of aconitase.

In conclusion, both the experiments on aconitase inhibition and those showing chromatographic and spectrophotometric evidence of the formation of the condensation compound seem to explain our previous results (D'Abramo *et al.* 1958), which showed the inhibition of citrate oxidation produced by the incubation with glyoxylate and oxaloacetate. A new compound is formed from the spontaneous reaction of glyoxylate plus oxaloacetate, and has been identified as the aconitase inhibitor. If this compound is a tricarboxylic acid with six carbon atoms (oxalomalic acid) it might inhibit citrate oxidation by competition with citrate for aconitase. The chemical nature of this compound is under investigation.

SUMMARY

1. Crude or purified aconitase preparations were inhibited after incubation in the presence of glyoxylate and oxaloacetate. Inhibition was demonstrated with amounts as low as 0·1 μmole of either glyoxylate or oxaloacetate in the presence of 4 μmoles of oxaloacetate or glyoxylate respectively.

2. Chromatographic evidence is given of the presence of a new compound formed after incubation of the purified enzyme with glyoxylate and oxaloacetate.

3. The presence of the same compound was chromatographically detected after incubation of glyoxylate and oxaloacetate in the presence of magnesium, but without enzyme. Spectrophotometric evidence for the disappearance of glyoxylate or oxaloacetate or both is given.

REFERENCES

Anfinsen, C. B. (1955). *Meth. Enzymol.* **1**, 695.
Cavallini, D. & Frontali, N. (1954). *Biochim. biophys. Acta*, **13**, 439.
Cavallini, D. & Mondovì, B. (1957). *Clin. chim. Acta*, **2**, 312.
D'Abramo, F., Romano, M. & Ruffo, A. (1957*a*). *Biochim. biophys. Acta*, **24**, 437.
D'Abramo, F., Romano, M. & Ruffo, A. (1957*b*). *Boll. Soc. ital. Biol. sper.* **33**, 1724.
D'Abramo, F., Romano, M. & Ruffo, A. (1958). *Biochem. J.* **68**, 270.
Ettinger, R. H., Goldbaum, L. R. & Smith, L. H. (1952). *J. biol. Chem.* **199**, 531.
Johnson, W. A. (1939). *Biochem. J.* **33**, 1046.
Stern, J. R. (1957). *Meth. Enzymol.* **3**, 425.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1951). *Manometric Methods and Tissue Metabolism*, p. 119. Minneapolis: Burgess Publishing Co.

The Production of Pyruvic Acid, Oxaloacetic Acid and α-Oxoglutaric Acid from Glucose by Tissue in Culture

By G. A. ABDEL-TAWAB,* E. BRODA
Erstes Chemisches Institut, Universität Wien, Austria
and G. KELLNER
Histologisch–Embryologisches Institut, Universität Wien, Austria

(*Received* 19 *January* 1959)

Glycolysis and respiration of cancer tissue *in vitro* have been the objects of many studies (see Warburg, 1956*a*, *b*; Weinhouse, 1955, 1956). Work with tissue in culture offers the general advantages that the cells are not only physiologically active, since in principle they proliferate indefinitely, but that they are also uniform. Therefore, the characteristic metabolism of cancer tissue is not 'diluted' by the metabolism of healthy tissue or modified by it as it is in intact animals. Moreover, the composition of the medium and the gas phase as well as the temperature can be varied at will within certain limits. However, the mass of tissue in culture is small (in many cases, 0·1–1 mg. dry weight/roller tube), and therefore sensitive methods are needed to determine the metabolites.

In these Laboratories, the production, from radioactive substrates, of certain metabolites by normal mesenchyma tissue and by HeLa tumour tissue has been determined in various experimental conditions (see Broda, Suschny & Kellner, 1958). Special attention was given to the mechanisms of energy production. The ratio of the amounts of radiocarbon in lactic acid (end-product of fermentation) and in carbon dioxide (end-product of respiration) after incubation of the tissue with radioglucose for a few hours or days was called the '*q* value' (Suschny, Kellner, Broda, Figdor & Rücker, 1958). The term 'fermentation' rather than 'glycolysis' is used here in view of the possibility that lactic acid arises through other pathways than that of Embden–Meyerhof–Parnas, e.g. through the pentose phosphate cycle.

Both with mesenchyma and HeLa tissue, the *q* values tend to increase when the tissues are exposed to certain damaging influences, e.g. treatment with trypsin, but they also increase with an improvement in the supply of glucose (Suschny *et al.* 1958). A shift from respiration to aerobic glycolysis with increasing supply of glucose is, of course, known from manometric experiments as the 'Crabtree effect' or—more frequently though misleadingly—as the 'inverse Pasteur effect'.

* On leave from the Chemistry Department, Teachers' College, Manshiet El-Bakry, Cairo, Egypt.

Significant differences between mesenchyma and HeLa tissue were found only in extreme conditions: when the supply of glucose was reduced to a very low level, and the *q* values consequently were brought down radically, only the normal tissue survived. With HeLa, no conditions were found where *q* was less than 1, i.e. where the utilization of glucose by respiration predominated over that by fermentation. On the other hand, the suppression of respiration by cyanide was, within a rather wide range of concentrations, tolerated by HeLa, but not by mesenchyma. Both results point to a shift in relative importance from respiration to aerobic glycolysis on the transition from a normal cell to a cancer cell.

In following up this line of work, it was important to determine the stationary concentrations of key intermediates of glycolysis and respiration in the same two kinds of tissue. Pyruvic acid, oxaloacetic acid and α-oxoglutaric acid were chosen. The concentrations of oxaloacetic acid and α-oxoglutaric acid relative to those of pyruvic acid should be depressed in tumour tissue if the operation of the citric acid cycle is partly inhibited. Again, the assay was done by radioactivity so that the amounts of the keto acids derived from (radioactive) glucose may be obtained. These amounts are exceedingly small.

In these experiments only about 0·1 μc of radiocarbon/ml. was applied. The resulting radiation dose, approx. 0·2 rep/day (equivalent to a γ-ray dose of 0·2 r./day), is not expected to damage the tissue to any noticeable extent, and in fact no damage has been observed. Damage has, however, been noted (in other experiments with tissue in culture) from doses of the order of 10 reps/day, as delivered by 1 μc of radiophosphorus/ml. (Kellner, Suschny, Broda & Rücker, 1959).

EXPERIMENTAL

The mesenchyma tissue ('fibroblasts') was obtained from legs of chick embryos 10 days old and grown in roller tubes at 37° as described previously (Kellner & Stockinger, 1957). The nutrient medium (1 ml./tube) consisted of 4% of

chick embryo extract, 40% of human ascitic fluid and 56% of Gey's buffered salt solution (8·0 g. of NaCl, 0·37 g. of KCl, 0·23 g. of $NaHCO_3$, 0·17 g. of $CaCl_2$, 0·21 g. of $MgCl_2$, 0·15 g. of Na_2HPO_4, 0·03 g. of KH_2PO_4, 0·07 g. of $MgSO_4$ and 1 g. of glucose/l.). (The glucose content of this mixture was very nearly 1 mg./ml.) About 4–5 days after explantation, the tissue in each tube was treated for 20 min. at pH 8·4 with 1 ml. of pancreatin solution with an activity equivalent to 0·11 mg. of crystallized trypsin to produce a cell suspension, the contents of the tubes were combined, the trypsin was removed by repeated washing with the buffered salt solution and the cells were resuspended in embryo extract and counted in a haemocytometer. A number of plasma-coated roller tubes were charged with 0·1 ml. of the suspension each, and incubated. Within a few hours, the cells settled on the wall as a monolayer. Now 1 ml. of nutrient medium was added and the incubation continued. At any time the cells could be counted again, in some of the tubes, after resuspending with trypsin. It was found that the cell counts decreased during the first day after the monolayer was formed; thereafter these counts increased. Monolayers of HeLa cells were made in an analogous manner from the HeLa tissue grown in these Laboratories for several years. The average dry weight of one HeLa cell is $7{\cdot}15 \times 10^{-10}$ g. (Leslie, Fulton & Sinclair, 1957), and the average weight of one of our chicken mesenchyma cells is about two-thirds of that value.

Randomly labelled radioglucose was obtained from Amersham and purified here by paper chromatography (Sverak, Suschny & Broda, 1953). Three days after the formation of the monolayer, the radioglucose was added along with 1 ml. of new nutrient medium, and incubation continued for 24 hr. Thereafter the metabolites were isolated, subjected to combustion by a wet method (Van Slyke & Folch, 1940) after addition of a known amount of inactive carrier, the radioactivity of the CO_2 measured with the gas Geiger counter (with almost 100% yield) for 16 min. and the activity referred to the total quantity of the metabolite; the measuring methods were the same as described before (Broda, 1955; Suschny *et al.* 1958). Whenever the original activity of the glucose was different from 0·1 μc/tube, the activities of the metabolites were calculated to this standard value.

In the work to be reported here, lactic acid and CO_2, as well as the three keto acids, were determined. The assay of the lactic acid and the CO_2 (Suschny *et al.* 1958) was always done on parallel but identical roller tubes; the detailed results will be given elsewhere (O. Suschny, G. A. Abdel-Tawab, G. Kellner, E. Broda & W. Rücker, in preparation), but some of the results will be used below. The numbers of cells per tube, at the end of each experiment, were also measured in parallel tubes. All determinations of metabolites or cell counts were done on three separate samples and the averages of these three independent results listed in the tables.

The principle of the isolation of the keto acids is as follows (Abdel-Tawab, Broda & Kellner, 1959); the tubes are cooled by ice immediately after incubation, the contents are homogenized and deproteinized and all carbonyl compounds are converted into the yellow 2:4-dinitrophenylhydrazones; these are extracted with ether, and the dinitrophenylhydrazones of the keto acids separated from other dinitrophenylhydrazones through the solubility of the former in alkali. Finally, the individual keto acid dinitrophenylhydrazones are isolated by descending chromatography on paper impregnated with 0·1 M-phosphate buffer, pH 7·4 (composed of 81·8 ml. of 0·1 M-Na_2HPO_4 and 18·2 ml. of 0·1 M-KH_2PO_4), with a solvent system consisting of butanol saturated with aq. 3% (w/v) NH_3. The dinitrophenylhydrazone of each keto acid is eluted from its spot and determined by colorimetry or by gas Geiger counting. Special colorimetric tests with known quantities of keto acids showed the yields to be at least 80%. As the radioactivities of the keto acids were rather low, the contents of several (as a rule, 4) parallel tubes were combined for isolation; however, the values in Table 1 are referred to single tubes.

It was necessary to find out whether the medium by itself, i.e. in absence of tissue, metabolized radioglucose. Therefore some blank tubes were incubated along with the tubes containing tissue, and the metabolites were isolated and their radioactivities measured. However, for CO_2 and the keto acids the blank values were always negligible (Suschny *et al.* 1958; Abdel-Tawab *et al.* 1959). Some small amounts of a radioactive acid behaving like lactic acid were found in the absence of tissue, and their radioactivity was deducted from that found in the presence of tissue; however, this correction was small.

In most experiments, the keto acids were determined separately within the tissue and within the medium. In this case the nutrient medium from each tube was decanted, the tissue quickly rinsed twice with 1 ml. of ice-cold saline (0·9% NaCl) solution, and the washings were combined with the decanted medium. Thereafter homogenization of the tissue, separate deproteinization of both tissue and supernatant medium, and separate treatment with 2:4-dinitrophenylhydrazine were carried out as described. It should be remembered, however, that with this procedure the keto acids within the plasma coating of the tubes are measured together with those actually within the tissue.

RESULTS

In Tables 1 and 2, the experiments with either mesenchyma or HeLa tissue are arranged in the order of decreasing numbers of cells per tube, i.e. of increasing supply of glucose per cell.

The radioactivities (Table 1) are given in 'disintegrations/min.' in the whole of the sample. The values listed were obtained, of course, by calculating, from the activities actually measured, the total amount of carbon in the sample (by multiplication) and that in a single tube (by division) (see Suschny *et al.* 1958). The lowest activities of the keto acids, accepted as significant, were 6 min.$^{-1}$ (per 4 combined tubes) above a background of 40 min.$^{-1}$. As all values given in the table are means of three independent determinations on parallel samples (4 tubes each), the standard deviation corresponding to this minimum activity is $\pm 1{\cdot}0$ min.$^{-1}$. Measured activities of 2 min.$^{-1}$ or less (per 4 tubes), again with an S.D. of $\pm 1{\cdot}0$ min.$^{-1}$, are considered as not significant, and entered in the table as zero. In fact, not a single measurement yielded an apparent activity of more than 2 but less than 6 min.$^{-1}$.

Table 1. *Radioactivities (per tube) of metabolites*

For details of incubation and extraction of cells, see Experimental section. For definition of *q*, see introduction. The cell counts refer to the end of the incubation, except for Expts. F1 and F3, in which they refer to the beginning of the incubation. The final cell counts are not known in Expts. F1 and F3; therefore the keto acids cannot be related to the cell counts.

Expt.	10^{-5} × cell count	Lactic acid + CO_2 (disintegrations/min.)	*q*	Pyruvic acid (disintegrations/min.)			Oxaloacetic acid (disintegrations/min.)			α-Oxoglutaric acid (disintegrations/min.)		
				Tissue	Medium	Total	Tissue	Medium	Total	Tissue	Medium	Total
					(*a*) Fibroblasts							
F1	15	11 900	1·2	—	—	628	—	—	24	—	—	57
F2	9, 54	29 100	5·7	38	291	329	17	0	17	19	0	19
F3	2, 5	39 200	15·8	—	—	385	—	—	25	—	—	20
F4	3, 81	35 500	17·5	30	790	820	20	0	20	20	0	20
					(*b*) HeLa							
H1	10	31 630	3·6	15	162	177	9	15	24	6	44	50
H3	4, 5	11 330	9·6	27	325	352	20	30	50	18	38	56
H2	2	15 130	10·2	12	105	117	7	11	18	8	38	46
H4	0, 7	3 460	19·2	33	288	321	23	49	72	20	34	54

In Table 2, the quantities of the keto acids are expressed in μg./10^7 cells of the glucose from which they were derived ('equivalent glucose'). For the purpose, the specific activity of the carbon of the keto acids at the time of isolation (i.e. after incubation for 24 hr.) is assumed to be the same as that of the carbon in the glucose. This assumption is safe, as much more radiocarbon was found in the two end-products of metabolism (lactic acid and CO_2) than in the keto acids; therefore, the mean life of the keto acids must have been comparatively small. For example, from a radioactivity of 3000 disintegrations/min. in the CO_2 accumulated by mesenchyma after 24 hr. and an activity of 20 disintegrations/min. in the stationary α-oxoglutaric acid, an average life of α-oxoglutaric acid of the order of a few minutes is obtained. Wherever a keto acid is found in two different pools, i.e. inside and outside the cells (e.g. for pyruvic acid), and is, no doubt, metabolized at different speeds in the two pools, a similar semiquantitative estimate is more difficult. It is clear, however, from the orders of magnitude involved that, in these cases as well, the specific activities of the carbon atoms in the keto acids must closely approach those of the glucose at the time of isolation.

DISCUSSION

The fate of the radioglucose in the tissue is identical with that of the total exogenous glucose. Moreover, for the following reason it must be almost identical with that of the total carbohydrate. The amount of carbohydrate in tissue is small (Šlechta, Jakubovič & Šorm, 1955; Willmer, 1954). Yet a fair fraction of the exogenous glucose is utilized during the experiment (usually 5–15 %, according to Table 1). This is possible only if the exogenous glucose rapidly penetrates the tissue and floods the endogenous carbohydrate.

As regards the keto acids, the following features emerge from the tables. Wherever the concentrations of oxaloacetic acid and α-oxoglutaric acid change, they do so in parallel. Apparently these changes reflect the role of the citric acid cycle as a whole. Thus there are no grounds to assume a specific block within the cycle which would cause an increase in the amount of some members at the expense of other members of the cycle.

The content of pyruvic acid per cell, in the mesenchyma or HeLa tissue, increases with a rise of the glucose supply. Some of the pyruvic acid diffuses into the medium. The total quantity of pyruvic acid in the medium is always higher than that within the tissue. However, as the volume of tissue plus plasma coating is so much less than that of the medium, the concentration of the pyruvic acid must always be less in the medium than in the tissue.

Table 2. *Stationary contents of keto acids*

Expt.	Pyruvic acid (PA) (μg./10^7 cells)			Oxaloacetic acid (OAA) (μg./10^7 cells)			α-Oxoglutaric acid (OGA) (μg./10^7 cells)			PA/OAA in tissue	PA/OGA in tissue
	Tissue	Medium	Total	Tissue	Medium	Total	Tissue	Medium	Total		
					(*a*) Fibroblasts						
F2	1·7	12·7	14·4	0·74	0	0·74	0·83	0	0·83	2·3	2·0
F4	3·3	86·7	90·0	2·2	0	2·2	2·2	0	2·2	1·5	1·5
					(*b*) HeLa						
H1	0·63	6·8	7·4	0·38	0·63	1·0	0·25	1·8	2·0	1·7	2·5
H3	2·5	30·1	32·6	1·9	2·8	4·7	1·7	3·5	5·2	1·3	1·5
H2	2·5	21·9	24·4	1·5	2·3	3·8	1·7	8	9·7	1·7	1·5
H4	19·5	169	189	13·5	29	42·5	12	20	32	1·4	1·6

In Table 2, the ratios between the amounts of pyruvic acid (PA), on the one hand, and of oxaloacetic acid (OAA) or α-oxoglutaric acid (OGA) on the other hand (PA/OAA or PA/OGA), are found to be $1{\cdot}6 \pm 0{\cdot}3$ and $1{\cdot}8 \pm 0{\cdot}4$ respectively. From the lack of an increase of this ratio with a rise in the glucose supply per cell, it can be concluded that in the given range the rate per molecule of the oxidative decarboxylation of pyruvic acid, followed by condensation with oxaloacetic acid and the reactions of the citric acid cycle, does not depend much on the concentration of glucose or pyruvic acid. In other words, the enzymes of the citric acid cycle (located in the mitochondria) do not lag appreciably behind the glycolytic enzymes (located in the soluble parts of the cytoplasm).

The pyruvic acid in the medium as well as in the tissue is acted upon by lactic dehydrogenase. True, the smallness of the blank values of lactic acid (after incubation of medium alone with radioglucose) indicates only low glycolytic activity of the medium. However, in contact with growing tissue the medium may take up glycolytic enzymes from dying cells, and reduced diphosphopyridine nucleotide by diffusion from intact cells as well. Thus the rise in pyruvic acid in the medium, caused by an increase in the supply of glucose per cell, may contribute a great deal to the increase in q, which is observed to be a consequence of this improved supply of glucose, and is the radiometric analogue of the manometric 'inverse Pasteur effect'.

A numerical comparison of the amounts, per cell, of lactic acid or CO_2 accumulated, or of the amounts, per cell, of the keto acids present in the stationary state, between mesenchyma and HeLa is not useful, as the two kinds of tissue were derived from different species. However, the ratios between the various metabolites, in the two kinds of tissue, can be compared.

The ratios PA/OAA and PA/OGA are not significantly different in mesenchyma and HeLa tissue. But there is a striking qualitative difference in that tumour tissue, in contrast with mesenchyma tissue, loses part of the two keto acids of the citric acid cycle into the medium. This can hardly be attributed to differences in permeability, although this remote possibility will be tested experimentally. It seems more likely that the two keto acids diffuse out of the tissue if they are not dealt with rapidly enough by the enzymes of the citric acid cycle, i.e. if the efficiency of the mitochondria is impaired.

At first glance, this result seems to be in contradiction with the approximate equality of the PA/OAA and PA/OGA ratios, or with that of the q values, between the two kinds of tissue. However, the loss of the keto acids from the tissue need not be reflected in an appreciable reduction of respiration. While the mean life of the keto acids is short within the tissue, diffused-out oxaloacetic acid and α-oxoglutaric acid can be metabolized only after re-entry into the tissue, i.e. after a relatively long time. From the similarity of the fates of the oxaloacetic acid and the (more stable) α-oxoglutaric acid within the medium it must also be concluded that little oxaloacetic acid only is lost by non-catalytic decarboxylation. Therefore even a small fractional loss of keto acids from the tissue will lead to a comparatively high stationary concentration within the medium.

SUMMARY

1. Monolayers of chick-embryo mesenchyma tissue and of human tumour tissue (HeLa) were incubated for 24 hr. with radioactive glucose. The metabolites lactic acid, carbon dioxide, pyruvic acid, oxaloacetic acid and α-oxoglutaric acid were isolated and their radioactivities measured with the gas Geiger counter.

2. When the supply of glucose per cell is raised, the ratio of radioactive lactic acid to radioactive carbon dioxide accumulated ('q value') increases sharply, indicating a shift from respiration to fermentation ('inverse Pasteur effect').

3. In stationary conditions, the quantities of the three keto acids can be calculated from their radioactivities. These quantities are small and correspond to the lactic acid or carbon dioxide generated during a few minutes only.

4. The average amount of each keto acid within a cell increases in parallel with the supply of glucose, but the ratio of the three keto acids does not change appreciably. Thus the enzymes of the citric acid cycle can, to a large extent, cope with an increase in the supply of pyruvic acid.

5. Pyruvic acid diffuses, from both mesenchyma and tumour tissue, into the medium where it is acted upon by lactic dehydrogenase. In the given conditions, the inverse Pasteur effect can partly be explained as being due to this reaction.

6. Oxaloacetic acid and α-oxoglutaric acid diffuse into the medium from tumour, but not from mesenchyma tissue. This loss is attributed to some malfunction of the mitochondria in tumour tissue. But it is shown that this malfunction need not express itself in any great increase of the ratio of pyruvic acid to oxaloacetic acid or α-oxoglutaric acid within the cells or any great decrease in the of rate respiration.

It is a pleasure to thank the Jane Coffin Childs Memorial Fund for Medical Research for generous financial support and Dr O. Suschny for useful advice. We wish to thank Mrs Abdel-Tawab for preparation of the text.

REFERENCES

Abdel-Tawab, G. A., Broda, E. & Kellner, G. (1959). *J. Chromat.* **2**, 99.
Broda, E. (1955). *J. inorg. Nuclear Chem.* **1**, 412.
Broda, E., Suschny, O. & Kellner, G. (1958). *Proc. 2nd Int. Conf. Peaceful Uses of Atomic Energy, Geneva*, **15** P, 1438.
Kellner, G. & Stockinger, L. (1957). *Arch. int. Pharmacodyn.* **110**, 259.
Kellner, G., Suschny, O., Broda, E. & Rücker, W. (1959). *Exp. Cell Res.* **6**, 673.
Leslie, I., Fulton, W. C. & Sinclair, R. (1957). *Biochim. biophys. Acta*, **24**, 365.
Šlechta, L., Jakubovič, A. & Šorm, F. (1955). *Chem. Listy*, **49**, 1706.
Suschny, O., Kellner, G., Broda, E., Figdor, B. & Rücker, W. (1958). *Exp. Cell Res.* **14**, 316.
Sverak, L., Suschny, O. & Broda, E. (1953). *Mh. Chem.* **84**, 921.
Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.
Warburg, O. (1956*a*). *Science*, **123**, 309.
Warburg, O. (1956*b*). *Science*, **124**, 269.
Weinhouse, S. (1955). *Advanc. Cancer Res.* **3**, 269.
Weinhouse, S. (1956). *Science*, **124**, 267.
Willmer, E. N. (1954). In *Tissue Culture*, p. 50. London: Methuen and Co.

Carbon Assimilation by *Pseudomonas oxalaticus* (OX 1)

1. FORMATE AND CARBON DIOXIDE UTILIZATION DURING GROWTH ON FORMATE

By J. R. QUAYLE and D. B. KEECH
Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford

(*Received* 9 *February* 1959)

Some non-photosynthetic micro-organisms can derive all their carbon and energy needs from oxalate or formate. It has been suggested that the organisms might obtain energy by oxidizing these compounds to carbon dioxide, and then synthesize cellular constituents from carbon dioxide (Bhat & Barker, 1948; Schatz, Isenberg & Trelawney, 1953; van Niel, 1954; Jayasuriya, 1955; Jakoby & Bhat, 1958). The isolation by Khambata & Bhat (1953) of *Pseudomonas oxalaticus* (OX 1), capable of growing on either formate or oxalate as the sole carbon and energy source, provided a suitable organism to investigate the reactions leading from these substrates to cell constituents. Results presented here and in the next paper are concerned with the carbon assimilation by *P. oxalaticus* (OX 1) grown on formate. A preliminary account of part of this work has been published (Quayle & Keech, 1958).

METHODS

Maintenance and growth of organism. Cultures of *Pseudomonas oxalaticus* (OX 1) (Khambata & Bhat, 1953) were obtained from the National Collection of Industrial Bacteria (Culture no. 8642) and maintained on slopes consisting of: 5 mM-potassium oxalate, 3 mM-sodium phosphate–potassium phosphate buffer, pH 7·0, essential salts [50 mg. of $(NH_4)_2SO_4$, 10 mg. of $MgSO_4,7H_2O$, 2 mg. of $FeSO_4,7H_2O$, 1 mg. of $CaSO_4$/100 ml. of medium], 0·1 % of yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.) and 1 % (w/v) of agar-agar (Davis, New Zealand). Stock cultures were grown at 30°, stored at 2° and subcultured every 4 weeks.

The medium used for liquid growth was the same as that used by Jayasuriya (1955) except that 100 mM-sodium formate was used as the carbon source. A portion (200 ml.) of this medium was inoculated with the organism from an agar slope and shaken in a Fernbach flask at 30° for approximately 48 hr. Growth was determined by measurements with a nephelometer (Evans Electroselenium Ltd., Essex) previously calibrated against dry weight of cells/ml. of bacterial suspension. The bacteria were harvested by centrifuging at room temperature while in the logarithmic growth phase at a cell density of 0·07–0·1 mg. dry wt./ml. (mean generation time was 3·5–5·0 hr.).

Manometric methods. Consumption of O_2 and CO_2 production were measured in the conventional Warburg manometric apparatus. For manometric experiments the washed bacteria were suspended in 0·9% KCl to a final concentration of approximately 2 mg. dry wt./ml. Generally 1 ml. of cell suspension was added to 100 μmoles of sodium phosphate–potassium phosphate buffer, pH 7·0, contained in the main compartment of the Warburg vessel, and 40 μmoles of substrate was added from the side arm.

Preparation of tracer solutions. Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks. By means of a transfer unit similar to that described by Aronoff (1956), barium [^{14}C]carbonate was decomposed with excess of 2N-$HClO_4$ and the liberated $^{14}CO_2$ trapped in the theoretical quantity of NaOH. The pH of the solution was adjusted finally to 7·5.

Sodium [^{14}C]formate was chromatographed on Whatman no. 4 filter paper with a solvent system of Long, Quayle & Stedman (1951) consisting of ethanol–aq. NH_3 soln. (sp.gr. 0·880)–water (80:4:16, by vol.). After development, the papers were sprayed with 0·5M-K_2HPO_4, dried in air and the formate area was located by radioautography. The radioactive area was eluted with water into one arm of a transfer unit (Aronoff, 1956), the formic acid was liberated with 5N-H_2SO_4 and distilled *in vacuo* into the theoretical quantity of NaOH solution.

Bacterial incorporation of [^{14}C]*formate and* [^{14}C]*bicarbonate.* Bacteria, which had been grown in Fernbach culture flasks, were centrifuged at room temperature and resuspended in a small volume of their supernatant medium. A sample of this suspension containing from 14 to 21 mg. dry wt. of bacteria was added to fresh growth medium (9 ml.) containing 10 mM-sodium formate (or 100 mM-sodium formate in the [^{14}C]bicarbonate experiment). The suspension was shaken in a 50 ml. Erlenmeyer flask at 30° for 10 min. (or 35 min. in the [^{14}C]bicarbonate experiment) to ensure that growth had re-commenced. At the end of this time, 200 μc of ^{14}C tracer solution was added to bring the final volume of the suspension to 10 ml. Samples (1 ml.) were withdrawn with a hypodermic syringe at suitable time intervals over 4 min., and transferred immediately to 3 ml. of boiling 95% (v/v) ethanol.

To study the incorporation under conditions of vigorous aeration, the above-described experiment was repeated with a cylindrical vessel (10 cm. × 2·5 cm. diam.) fitted with a sintered-glass bottom. Vigorous aeration in this suspension was maintained by blowing a stream of air through the sintered glass.

A comparison was made of [^{14}C]formate incorporation into bacteria growing in shaken cultures in air and in air + CO_2 (95:5, v/v). In this experiment, two cultures of the organism were harvested while in the logarithmic phase of growth. The cells were resuspended in two Fernbach flasks, each containing 200 ml. of fresh growth medium, at a cell density of about 0·06 mg. dry wt./ml.; the substrate in each flask was 100 mM-sodium [^{14}C]formate, containing 200 μc of ^{14}C. Samples (20 ml.) of the cultures were withdrawn at intervals, their growth was measured nephelometrically and then they were centrifuged at 0°. The supernatant medium was poured off and the cells were resuspended in 0·5 ml. of water; samples (0·2 ml.) were immediately plated on to glass planchets (3 cm.^{2} area) on a rotating turntable in a stream of warm air. Each planchet was washed with 0·1 ml. of formic acid (90%, w/v; A.R.), dried and the radioactivity assayed with a mica end-window, lead-shielded G.M. tube (General Electric Co. Ltd.) A blank determination showed that a negligible amount of radioactive formate remained on the planchets after this treatment. A coincidence correction was applied to the counts obtained.

Treatment of aqueous ethanolic suspensions of killed cells, and radioassay. The hot ethanolic suspensions described previously were centrifuged and the resulting precipitates washed with 1 ml. of hot aqueous 20% (v/v) ethanol. The combined supernatant fractions were evaporated to dryness at 60° under N_2 and redissolved in aqueous 20% (v/v) ethanol ready for application to the origin positions of paper chromatograms. The ethanol-insoluble fractions were freed from nucleic acid contamination by a procedure similar to that of Roberts, Abelson, Cowie, Bolton & Britten (1955), by suspension in 2 ml. of 5% (w/v) trichloroacetic acid at 100° for 30 min. The resulting insoluble protein residues were washed with 1 ml. of acidified ethanol, re-centrifuged and hydrolysed in 0·5 ml. of 6N-HCl at 110° for 15 hr. in sealed tubes. The total radioactivity contained in the protein hydrolysates was measured by evaporation, at 60°, of 10–250 μl. samples on silicone-treated glass coverslips (21 mm. diam.) supporting circles of lens tissue. The tissues were secured by means of 0·05 ml. of 10% (w/v) mannitol solution.

The remainder of the protein hydrolysate was analysed chromatographically for labelled amino acids. Each sample was evaporated at 80° under N_2; the residue was resuspended in 0·5 ml. of water and re-evaporated. The resulting residue was finally taken up in 0·25 ml. of water and chromatographed in a similar manner to the ethanol-soluble fraction.

Chromatographic analysis and radioautography. The solutions prepared from either the ethanol-soluble or ethanol-insoluble fractions of the reaction mixture were applied to chromatographic paper (Whatman no. 4, 18½ in. × 22 in.) and chromatographed two-dimensionally in a descending direction. The chromatogram was developed first across the machine direction of the paper with the solvent system of Kornberg (1958): 500 g. of phenol–13 ml. of 90% (w/v, A.R.) formic acid–167 ml. of water. The solvent system butanol–propionic acid–water (47:22:31, by vol.; Benson *et al.* 1950) was used to develop the chromatogram in the second dimension. Both solvents were allowed to run to the edge of the paper.

Radioautographs were made from the chromatograms by the procedure developed by Calvin and his co-workers (see Calvin, 1950–51), with Blue Brand X-ray film (Kodak Ltd.). The radioactive areas located on the paper were counted directly with a mica end-window G.M. tube (General Electric Co., type 2B2) and the count was corrected for

Table 1. *Oxidation of formate by washed suspensions of* Pseudomonas oxalaticus

Manometer cups contained, in the main compartment, washed bacteria (2·4 mg. dry wt.) in 42 mM-sodium phosphate, pH 7·0, and inhibitor where indicated; the side arm contained substrate; the centre well contained 400 μmoles of KOH. Total volume of contents, 3 ml. Incubation was at 30° for 1 hr. The results in parentheses refer to a second experiment performed on a different batch of cells.

	Cells alone		Cells + 1·1 mM-DNP		O_2 uptake by cells + 1 mM-NaH_2PO_2 (μl.)
Substrate	O_2 uptake (μl.)	CO_2 output (μl.)	O_2 uptake (μl.)	CO_2 output (μl.)	
(*a*) None	31 (32)	41 (40)	32	33	—
(*b*) Formate (30 μmoles)	258 (230)	480 (480)	324	543	0
Theoretical values*	336	672	336	672	—
Q_{O_2}	−270 (−144)	—	−310	—	0
R.Q. observed	1·87 (2·10)		1·68		—
R.Q. theoretical	2·00		2·00		—

* Theoretical values for complete oxidation of formate to CO_2.

background activity. The relationship between counting a sample of radioactive glucose mounted on a lens-tissue planchet (see above) and the same sample counted *in situ* after paper chromatography was found to be (planchet counts: paper counts) 1·5:1.

Identification of labelled compounds. The radioactive compounds were eluted from the chromatogram with water and co-chromatographed in appropriate solvent systems with authentic samples of the suspected compound. Exact matching of the resulting radioactive area and the coloured area developed with an appropriate spray reagent was necessary for identification. Carboxylic acids were re-chromatographed either in the original solvent systems or in ethanol–aq. NH_3 soln. (sp.gr. 0·880)–water (80:4:16, by vol.; Long *et al.* 1951). Amino acids were rechromatographed in ethanol–butanol–water (10:10:5, by vol.; Hardy, Holland & Nayler, 1955) or water-saturated phenol in the presence of NH_3 vapour (Consden, Gordon & Martin, 1944). The latter solvent was found particularly useful for the separation of aspartate, glutamate, serine and glycine. Phosphorylated compounds were rechromatographed two-dimensionally in methanol–90% formic acid–water (80:15:5, by vol.) and methanol–aq. 35% (w/v) NH_3 soln.–water (60:10:30, by vol.; Bandurski & Axelrod, 1951). Dephosphorylation was carried out by incubation of the eluted compounds with Polidase-S (Schwarz Laboratories, Inc., New York, U.S.A.) according to the procedure of Benson, Bassham & Calvin (1951) and the resulting compounds were chromatographed two-dimensionally in water-saturated phenol and butanol–propionic acid–water (47:22:31, by vol.; Benson *et al.* 1950).

Sprays. Carboxylic acids were located on chromatograms by spraying with ethanolic 50% (v/v) Universal Indicator (British Drug Houses Ltd., Poole, Dorset) adjusted to pH 10; amino acids by spraying with 0·2% (w/v) ninhydrin in butanol–2N-acetic acid (95:5, v/v); phosphorylated compounds by first spraying with 0·1% (w/v) $FeCl_3,6H_2O$ in 80% (v/v) ethanol followed by 1% (w/v) salicylsulphonic acid in 80% (v/v) ethanol (Wade & Morgan, 1953). Aldose sugars were detected with aniline–phthalic acid (0·9:1·7, w/v) in 100 ml. of water-saturated butanol, followed by heating to 105° for 5 min. (Partridge, 1949); fructose was detected by spraying with ethanolic 1% (w/v) naphtharesorcinol–2N-HCl (10:90, v/v; Forsyth, 1948) followed by heating to 90° for 10 min.; sedoheptulose by spraying with a solution of orcinol–trichloroacetic acid (0·5:15, w/v) in 100 ml. of water-saturated butanol, followed by heating at 100° (Klevstrand & Nordal, 1950).

RESULTS

Oxidation of formate. Washed suspensions of *P. oxalaticus* (OX 1) oxidized formate with Q_{O_2} values of −140 to −270 (μl. of O_2 consumed/mg. dry wt. of bacteria/hr.; Table 1). The oxidation was completely inhibited in the presence of mM-sodium hypophosphite, a compound which is an inhibitor of formic dehydrogenase in some organisms (Takamiya, 1953; Campbell, 1954, 1955). Although different batches of cells gave respiratory quotients for this substrate ranging from 1·87 to 2·10 (theoretical value, 2·0), the volumes of O_2 consumed and CO_2 evolved were always below those required for complete oxidation. Values of 68–80% of the theoretical were generally obtained, which suggested an oxidative assimilation of a portion of the substrate.

In the presence of mM-2:4-dinitrophenol (DNP), the uptake of O_2 was raised to 96% of the theoretical value and the respiratory quotient dropped to 1·7 (Table 1). The increased uptake of O_2 observed in the presence of DNP, which is known to be an inhibitor of oxidative assimilation (Clifton, 1937), supports the suggestion that the incomplete oxidation of formate by washed organisms was due to assimilation of the substrate. In these calculations, gas exchanges observed in the absence of substrate were not subtracted from those observed in its presence.

Incorporation of [^{14}C]*formate and* [^{14}C]*bicarbonate.* Addition of either [^{14}C]formate or [^{14}C]bicarbonate to growing cultures resulted in incorporation of isotope into both the ethanol-soluble and protein fractions of the cells (Fig. 1).

The incorporation of [^{14}C]formate was studied under conditions of vigorous aeration in the

bubbler flask and the [^{14}C]bicarbonate experiment was conducted in a shaken Erlenmeyer flask to avoid any possible sweeping out of the isotope. Separate experiments had shown that the growth rates of the organism under these two conditions were not significantly different. The observed average incorporation rates of [^{14}C]formate and [^{14}C]bicarbonate carbon into the ethanol-soluble fractions of the cells were 1620 and 354 counts/min./mg. dry wt. of bacteria respectively. The specific activity of the [^{14}C]formate tracer used was four times that of the [^{14}C]bicarbonate, hence the observed incorporation rates in terms of μg.atoms of carbon from either formate or bicarbonate are similar. However, since small quantities of both unlabelled formate and bicarbonate would be present at the start of the experiments, a very accurate comparison of the two incorporation rates cannot be made, owing to the resulting uncertainty of the initial specific activity of the tracers in the bacterial suspensions.

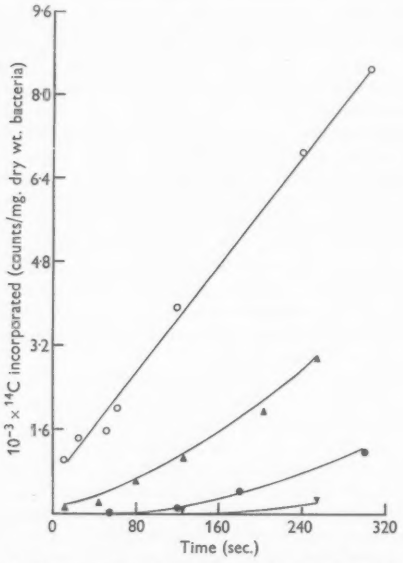


Fig. 1. Incorporation of ^{14}C from [^{14}C]formate and [^{14}C]-bicarbonate into the protein and ethanol-soluble fractions of *Pseudomonas oxalaticus*. ○, Ethanol-soluble fractions; ●, protein fractions; conditions were as described in Table 2 ([^{14}C]formate incorporation). ▲, Ethanol-soluble fractions; ▼, protein fractions; conditions were as described in Table 3 ([^{14}C]bicarbonate incorporation). All the counts in the protein fractions have been reduced by a factor of 0·665 to correct for the different method of radioassay used from that used for the ethanol-soluble fraction.

Distribution of radioactivity amongst labelled compounds in the ethanol-soluble fractions. Chromatographic analysis, followed by radioautography, showed that radioactivity either from [^{14}C]formate or [^{14}C]bicarbonate very rapidly appeared in a large number of compounds; over 30 labelled compounds in the ethanol-soluble fraction were visible after incubation for 90 sec. with [^{14}C]formate under the conditions of high aeration. The distribution of radioactivity amongst the labelled compounds formed during the first 90 sec. incubation with [^{14}C]formate or [^{14}C]-bicarbonate is shown in Tables 2 and 3. Under the conditions of chromatography used, phosphorylated compounds ran together in one large spot near the origin of the chromatograms. The earliest time samples showed radioactivity only in this phosphate area and, to a minor extent, in malic acid. In the later samples radioactivity progressively appeared also in glutamic acid, aspartic acid and citric acid. The curves obtained by plotting time against the percentage of the total radioactivity contributed by each constituent of the sample are shown in Figs. 2 and 3. The most striking feature of both diagrams is the very high percentage of total radioactivity appearing in the earliest times in phosphorylated compounds. Furthermore, the negative slopes of these curves show that a primary fixation product was present in these compounds. Malate was labelled in both cases at the earliest times, to the extent of about 5 % of the total radioactivity. This figure rose slightly during the course of the incubation. These results show that isotope from either [^{14}C]formate or [^{14}C]bicarbonate was incorporated by the cells via phosphorylated compounds.

The composition of the phosphate areas on the chromatograms is shown in Table 4. Losses of count from the original phosphate area were always observed (compare Table 2); part of this may have been due to irreversible absorption of the phosphates on the paper and part to the loss of volatile compounds after dephosphorylation (e.g. pyruvic acid). In the earliest samples, 81 % of the recovered radioactivity in the dephosphorylated compounds was contained in glyceric acid, the remainder in glucose, fructose and sedoheptulose. Ribose became labelled in the later samples, although the amount of radioactivity incorporated into this compound was surprisingly low.

The composition of the phosphate areas in the [^{14}C]bicarbonate experiment was investigated by rechromatographing two-dimensionally in the solvent system of Bandurski & Axelrod (1951) (Table 5). Considerable losses in total count recovery were again encountered, but the only labelled compound that appeared in the phosphate chromatogram from the earliest sample was 3-phosphoglyceric acid (PGA).

Table 2. *Distribution of radioactive carbon amongst the non-volatile labelled components of the ethanol-soluble fractions of growing suspensions of* Pseudomonas oxalaticus *incubated with sodium* [^{14}C]*formate*

Sodium [^{14}C]formate (47 μmoles; 200 μc of ^{14}C) was added at zero time to 10 ml. of a suspension of bacteria (21 mg. dry wt.) growing with vigorous aeration on 10 mM-sodium formate, 25 mM-potassium phosphate, pH 7·0, and essential salts. Samples (1 ml.) were withdrawn by hypodermic syringe at known times and transferred into 3 ml. of hot ethanol. The precipitates were each washed with 1 ml. of hot aqueous 20% (v/v) ethanol and analysed by two-dimensional chromatography. The radioactive areas were located by radioautography and directly counted with a mica end-window G.M. tube.

	Radioactivity (counts/min.)						
Time (sec.)	Phosphorylated compounds	Malate	Glutamate	Aspartate	Citrate	Other compounds	Total
12	623	35	0	0	0	130	788
25	1358	104	0	0	0	268	1730
53	2520	200	153	64	0	412	3349
63	2410	180	264	88	37	660	3639
91	3130	323	367	163	182	700	4865

Table 3. *Distribution of radioactive carbon amongst the non-volatile labelled components of the ethanol-soluble fractions of growing suspensions of* Pseudomonas oxalaticus *incubated with sodium* [^{14}C]*bicarbonate*

Sodium [^{14}C]bicarbonate (190 μmoles; 200 μc of ^{14}C) was added at zero time to 10 ml. of a suspension of bacteria (14·7 mg. dry wt.) growing in a 50 ml. Erlenmeyer flask on 100 mM-sodium formate, 25 mM-potassium phosphate, pH 7·0, and essential salts. Samples (1 ml.) were withdrawn by hypodermic syringe at known times and treated as described in Table 2.

	Radioactivity (counts/min.)						
Time (sec.)	Phosphorylated compounds	Malate	Glutamate	Aspartate	Citrate	Other compounds	Total
13	162	8	0	0	0	0	170
45	280	34	0	0	0	38	352
62	484	53	53	9	0	21	620
80	514	69	98	15	8	82	786

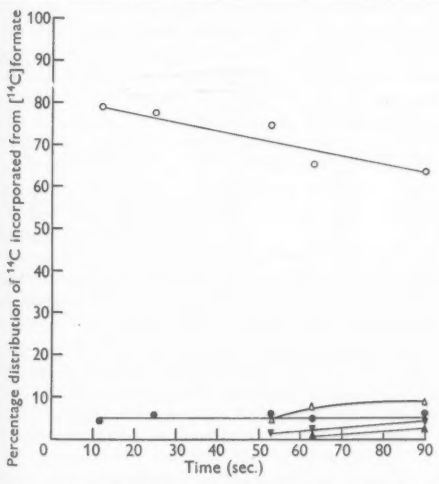


Fig. 2. Variation with time of the percentage distribution of ^{14}C from [^{14}C]formate incorporated into the constituents of the ethanol-soluble fraction of *Pseudomonas oxalaticus* growing on formate. Conditions were as described in Table 2. ○, Phosphorylated compounds; ●, malate; △, glutamate; ▲, citrate; ▼, aspartate.

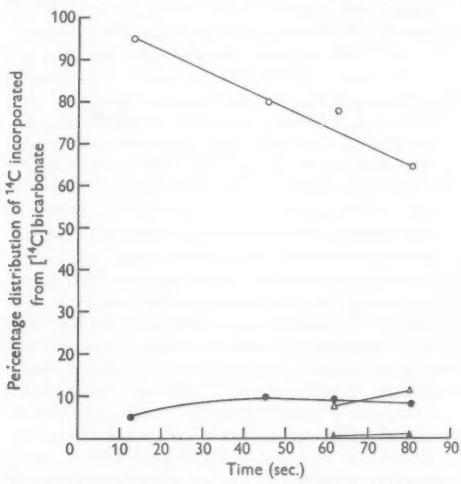


Fig. 3. Variation with time of the percentage distribution of ^{14}C from [^{14}C]bicarbonate incorporated into the constituents of the ethanol-soluble fraction of *Pseudomonas oxalaticus* growing on formate. Conditions were as described in Table 3. ○, Phosphorylated compounds; ●, malate; △, glutamate; ▲, aspartate.

Table 4. *Distribution of radioactive carbon amongst the labelled phosphates obtained from the ethanol-soluble fractions of growing suspensions of* Pseudomonas oxalaticus *incubated with sodium* [^{14}C]*formate*

The phosphorylated compounds were eluted from the chromatograms described in Table 2, and the eluates concentrated to 0·5 ml. and incubated overnight at 30° with 0·2 mg. of Polidase S. The resulting non-volatile dephosphorylated compounds were analysed by two-dimensional paper chromatography and located by radioautography.

	Radioactivity (counts/min.)					
Time (sec.)	Glyceric acid	Glucose and sedoheptulose	Fructose	Ribose	Other compounds	Total
12	373	73	13	0	0	459
25	263	372	63	<10	110 (Chromatogram origin)	818
53	358	600	130	<10	30	1128
63	394	593	150	<10	400 (Chromatogram origin)	1547
91	476	600	25	<10	35	1146

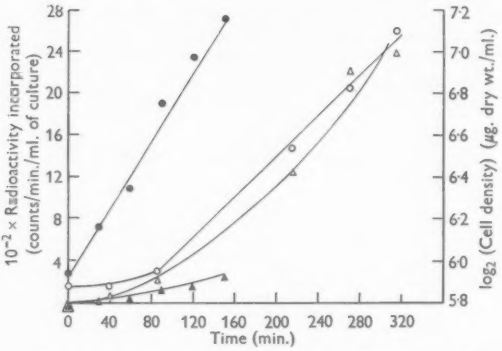


Fig. 4. Incorporation of ^{14}C from [^{14}C]formate into *Pseudomonas oxalaticus* growing in air and in air + CO_2 (95:5) at 30°. ○, Growth in air; ●, growth in air + CO_2; △, radioactivity incorporated by air-grown culture; ▲, radioactivity incorporated by culture grown in air + CO_2.

Distribution of radioactivity amongst labelled compounds in protein hydrolysates. The first amino acid to become labelled in the protein fraction of cells growing on [^{14}C]formate was aspartic acid (Table 6). Glutamate and alanine followed, with radioactivity also appearing in three spots running closely together in the leucine area of the chromatogram. These compounds were not identified.

Incorporation of [^{14}C]*formate in the presence and absence of added carbon dioxide.* The growth rate of *P. oxalaticus* was higher (mean generation time 2 hr.) during growth in air + CO_2 (95:5) than it was (mean generation time 3·5 hr.) in air alone (Fig. 4). This could be due either to the higher concentration of CO_2 or to the establishment of an efficient buffer system. The total incorporation of ^{14}C into the cells under these two conditions was

Table 5. *Distribution of radioactive carbon amongst the labelled phosphates obtained from the ethanol-soluble fractions of growing suspensions of* Pseudomonas oxalaticus *incubated with sodium* [^{14}C]*bicarbonate*

The phosphorylated compounds were eluted from the chromatograms described in Table 3 and the eluates treated with Dowex-50 (H^+ form), analysed by two-dimensional paper chromatography and the phosphates located by radioautography. PGA was identified by co-chromatography with an authentic sample, and the sugar phosphates were eluted, dephosphorylated and identified chromatographically as described in Table 4.

	Radioactivity (counts/min.)			
Time (sec.)	PGA	Glucose phosphate, fructose phosphate and sedoheptulose phosphate	Other compounds	Total
13	67	0	0	67
45	70	<10	8	88
62	49	84	46	179
80	90	112	86	288

Table 6. *Distribution of radioactive carbon in the protein hydrolysate of* Pseudomonas oxalaticus *incubated with sodium* [^{14}C]*formate*

The ethanol-insoluble fractions from Table 2 were heated in 2 ml. of 5% (w/v) trichloroacetic acid at 100° for 30 min. Insoluble residues were washed with 1 ml. of acidified ethanol and hydrolysed in 0·5 ml. of 6N-HCl at 110° for 15 hr. Samples were analysed by chromatography and radioautography as described in Table 2.

	Radioactivity (counts/min.)			
Time (sec.)	Aspartate	Glutamate	Alanine	Other compounds
91	66	0	0	0
180	263	122	0	237
304	300	288	244	1200

measured in each case over one complete generation, the range of cell densities being the same in both cases (Fig. 4).

Growth of the air-grown cells from a cell density of 0·063 mg. dry wt./ml. to 0·126 mg. dry wt./ml. was accompanied by an incorporation of radioactivity of 2990 counts/min./ml. of culture; that of the culture gassed with CO_2 was accompanied by an incorporation of 180 counts/min./ml. of culture. This shows that at least 94 % $\left(100-\frac{180\times100}{2990}\right)$ of the carbon entering the cell from formate passes through the stage of CO_2 or a compound which is in rapid equilibrium with extracellular CO_2.

DISCUSSION

The ability of some non-photosynthetic micro-organisms to exist on C_1 compounds as combined energy and growth substrates has been known for a long time. Amongst the well-characterized species may be mentioned those living by oxidation of methane (Hutton & ZoBell, 1949), formate (Kingma Boltjes, 1936; Mevius, 1953; Khambata & Bhat, 1953, 1955) and the methane fermentation of formate (Stadtman & Barker, 1951). The assimilation of their cellular carbon by carbon dioxide fixation, as previously suggested, would make such organisms analogous to the autotrophic bacteria, which live at the expense of the oxidation of inorganic substances. The difference between the two processes would thus be one of energy source only, being an organic and inorganic oxidation respectively. Some evidence for this type of metabolism of an organic substrate has been obtained by Schatz *et al.* (1953) with *Hydrogenomonas facilis*. Schatz *et al.* found that, when *H. facilis* oxidized lactate in the presence of [^{14}C]carbon dioxide: 'the CO_2...assimilated was far greater than could be accounted for by the ordinary heterotrophic fixation'.

Studies of carbon dioxide fixation by cell-free extracts of the autotrophic bacteria *Thiobacillus thioparus* (Santer & Vishniac, 1955), *T. denitrificans* (Trudinger, 1955, 1956), *T. thio-oxidans* (Suzuki & Werkman, 1958) and *Hydrogenomonas ruhlandii* (Santer & Vishniac, 1957) have shown the ability of the extracts to carry out enzymic carboxylation of a phosphorylated pentose to give PGA. Furthermore, a kinetic analysis of the distribution of radioactivity among the intermediates formed from [^{14}C]carbon dioxide by *T. denitrificans* (Aubert, Milhaud & Millet, 1956, 1957; Milhaud, Aubert & Millet, 1956), *T. thio-oxidans* (Suzuki & Werkman, 1957) and *H. facilis* (Bergmann, Towne & Burris, 1958) indicates that the growth of these organisms depends on the operation of a carbon dioxide-fixation cycle similar to that found in photosynthetic organisms (Bassham *et al.* 1954).

The alternative to this autotrophic mechanism in organisms growing on C_1 organic compounds would be the assimilation of a C_1 unit through an unknown series of condensation reactions. Such reactions would have to accomplish an overall chemical reduction when the carbon source is at a higher oxidation level than that of cell carbon.

The finding that PGA and, to a minor extent, malate, were the first labelled compounds to be formed from either [^{14}C]formate or [^{14}C]bicarbonate by *P. oxalaticus* grown on formate suggests that the main pathway of carbon assimilation into cellular constituents is via carboxylation of ribulose 1:5-diphosphate, together with a minor incorporation into malate (or possibly oxaloacetate as well). The early labelling of phosphate esters of glucose, fructose, sedoheptulose and ribose is consistent with the operation of a ribulose 1:5-diphosphate-regenerating cycle. The appearance of aspartate as the first labelled amino acid residue in the protein fractions would follow from carboxylation of pyruvate or of phosphopyruvate or of both, the two compounds being derived from radioactive PGA.

The assimilation of formate carbon into the cell by these means necessitates carbon dioxide being an intermediary metabolite, unless in these organisms ribulose 1:5-diphosphate can react directly with formate. No such reaction could be demonstrated with cell-free extracts (Quayle & Keech, 1959). Furthermore, the fact that at least 94 % of the carbon assimilated by the cell was exchangeable with carbon dioxide indicates that assimilation of formate is preceded by oxidation to carbon dioxide.

These findings contrast with those obtained with *Pseudomonas* KB1 grown on [^{14}C]acetate (Kornberg & Quayle, 1958), where it was found that cells growing in a stream of oxygen+carbon dioxide (95:5) synthesized protein of specific radioactivity 15–17 % less than that synthesized in a stream of oxygen. The deduction that a maximum of 15–17 % of the carbon incorporated into the cell passes through the stage of carbon dioxide has been criticized on the grounds of insufficiently rapid equilibration between intra- and extra-cellular carbon dioxide. The findings with *P. oxalaticus* tend to invalidate this criticism.

The results reported in this paper therefore show that a major pathway of carbon incorporation from formate into cellular constituents occurs by a carbon dioxide-fixation cycle, similar to that found in photosynthetic organisms and other autotrophic organisms. Studies carried out with cell-free extracts of *P. oxalaticus* confirm this conclusion (Quayle & Keech, 1959).

SUMMARY

1. The incorporation of carbon from either [^{14}C]formate or [^{14}C]bicarbonate by cultures of *Pseudomonas oxalaticus* (OX 1) grown on formate has been studied.

2. Isotope appeared in both the aqueous ethanol-soluble and protein fractions of the cells during 5 min. incubation periods.

3. The distribution of radioactivity within the non-volatile constituents of these fractions, after incubation for from 12 sec. to 5 min., has been analysed by chromatography and radioautography. Over 80 % of the radioactivity incorporated at the earliest time (12 sec.) into the ethanol-soluble fractions was present in phosphorylated compounds, and 5 % in malate, with either [^{14}C]formate or [^{14}C]bicarbonate. After incubation for 1 min., radioactivity also appeared in citrate, aspartate and glutamate.

4. In 12 sec., over 80 % of the radioactivity initially incorporated into phosphorylated compounds was present in 3-phosphoglyceric acid; phosphates of glucose, fructose, sedoheptulose and ribose (to a minor extent) became labelled in the later samples.

5. The specific radioactivity of cellular material obtained from cells grown on [^{14}C]formate in air was reduced 17-fold by bubbling air + carbon dioxide (95:5, v/v) through the growing culture. Thus at least 94 % of the carbon incorporated must have passed through the stage of carbon dioxide or a compound in ready equilibrium with it.

6. These results show that the organism synthesizes cell constituents from carbon dioxide by a cycle similar to that found in photosynthetic organisms and other autotrophic organisms. The necessary energy is derived from oxidation of the formate to carbon dioxide.

We wish to thank Professor Sir H. A. Krebs, F.R.S., for his interest and encouragement, Mrs M. Jenkins for her skilled technical assistance and Mr D. Peel for measuring the specific radioactivity of cellular material grown under different gassing conditions. We also wish to acknowledge many helpful discussions with colleagues, especially Dr D. E. Hughes and Dr H. L. Kornberg. This work was supported by the Rockefeller Foundation and by the office of Scientific Research of the Air Research and Development Command of the United States Air Force, through its European Office, under contract no. AF. 61 (514)–1180, and was done while one of us (D.B.K.) held an Overseas Scholarship of the Commonwealth Scientific and Industrial Research Organization (Australia).

REFERENCES

Aronoff, S. (1956). *Techniques of Radiobiochemistry*, p. 71. Iowa: Iowa State College Press.

Aubert, J. P., Milhaud, G. & Millet, J. (1956). *C.R. Acad. Sci., Paris*, **242**, 2059.

Aubert, J. P., Milhaud, G. & Millet, J. (1957). *Ann. Inst. Pasteur*, **92**, 515.

Bandurski, R. S. & Axelrod, B. (1951). *J. biol. Chem.* **193**, 405.

Bassham, J. A., Benson, A. A., Kay, L. D., Harris, A. E., Wilson, A. T. & Calvin, M. (1954). *J. Amer. chem. Soc.* **76**, 1760.

Benson, A. A., Bassham, J. A. & Calvin, M. (1951). *J. Amer. chem. Soc.* **73**, 2970.

Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A. & Stepka, W. (1950). *J. Amer. chem. Soc.* **72**, 1710.

Bergmann, F. H., Towne, J. C. & Burris, R. H. (1958). *J. biol. Chem.* **230**, 13.

Bhat, J. V. & Barker, H. A. (1948). *J. Bact.* **55**, 359.

Calvin, M. (1950–51). *Harvey Lect.* **46**, 218.

Campbell, L. L. (1954). *J.Bact.* **68**, 598.

Campbell, L. L. (1955). *J. biol. Chem.* **217**, 669.

Clifton, C. E. (1937). *Enzymologia*, **4**, 246.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.

Forsyth, W. G. C. (1948). *Nature, Lond.*, **161**, 239.

Hardy, T. L., Holland, D. O. & Nayler, J. H. C. (1955). *Analyt. Chem.* **27**, 971.

Hutton, W. E. & ZoBell, C. E. (1949). *J. Bact.* **58**, 463.

Jakoby, W. B. & Bhat, J. V. (1958). *Bact. Rev.* **22**, 75.

Jayasuriya, G. C. N. (1955). *J. gen. Microbiol.* **12**, 419.

Khambata, S. R. & Bhat, J. V. (1953). *J. Bact.* **66**, 505.

Khambata, S. R. & Bhat, J. V. (1955). *J. Bact.* **69**, 227.

Kingma Boltjes, T. Y. (1936). *Arch. Mikrobiol.* **7**, 188.

Klevstrand, R. & Nordal, A. (1950). *Acta chem. scand.* **4**, 1320.

Kornberg, H. L. (1958). *Biochem. J.* **68**, 535.

Kornberg, H. L. & Quayle, J. R. (1958). *Biochem. J.* **68**, 542.

Long, A. G., Quayle, J. R. & Stedman, R. J. (1951). *J. chem. Soc.* p. 2197.

Mevius, W. (1953). *Arch. Mikrobiol.* **19**, 1.

Milhaud, G., Aubert, J. P. & Millet, J. (1956). *C.R. Acad. Sci., Paris*, **243**, 102.

Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.

Quayle, J. R. & Keech, D. B. (1958). *Biochim. biophys. Acta*, **29**, 223.

Quayle, J. R. & Keech, D. B. (1959). *Biochem. J.* **72**, 631.

Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Studies of Biosynthesis in* Escherichia coli, p. 14. Washington, D.C.: Carnegie Institution.

Santer, M. & Vishniac, W. (1955). *Biochim. biophys. Acta*, **18**, 157.

Santer, M. & Vishniac, W. Unpublished observation quoted in *Advanc. Enzymol.* (1957). **19**, 1.

Schatz, A., Isenberg, H. D. & Trelawney, G. S. (1953). *Bact. Proc.* p. 88.

Stadtman, T. C. & Barker, H. A. (1951). *J. Bact.* **62**, 269.

Suzuki, I. & Werkman, C. H. (1957). *Bact. Proc.* p. 120.

Suzuki, I. & Werkman, C. H. (1958). *Arch. Biochem. Biophys.* **77**, 112.

Takamiya, A. (1953). *J. Biochem., Tokyo*, **40**, 415.

Trudinger, P. A. (1955). *Biochim. biophys. Acta*, **18**, 581.

Trudinger, P. A. (1956). *Biochem. J.* **64**, 274.

van Niel, C. B. (1954). *Annu. Rev. Microbiol.* **8**, 105.

Wade, H. E. & Morgan, D. M. (1953). *Nature, Lond.*, **171**, 529.

Carbon Assimilation by *Pseudomonas oxalaticus* (OX 1)

2. FORMATE AND CARBON DIOXIDE UTILIZATION BY CELL-FREE EXTRACTS OF THE ORGANISM GROWN ON FORMATE*

By J. R. QUAYLE and D. B. KEECH
Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford

(*Received 9 February* 1959)

Isotopic experiments performed on cultures of *Pseudomonas oxalaticus* (OX 1) growing on formate as sole carbon and energy source (Quayle & Keech, 1958, 1959) indicated the existence in the organism of a carbon dioxide-fixation cycle similar to that found in photosynthetic tissue (Bassham *et al.* 1954) and autotrophic bacteria (Santer & Vishniac, 1955; Trudinger, 1955).

An investigation of the ability of cell-free extracts of *P. oxalaticus* (OX 1) to incorporate formate carbon and carbon dioxide into 3-phosphoglyceric acid and certain C_4 acids is reported in this paper.

METHODS AND MATERIALS

Maintenance and growth of the organism. The maintenance and growth of stock cultures of *Pseudomonas oxalaticus* (OX 1) have been described (Quayle & Keech, 1959). Large quantities of the organism were grown in 40 l. of medium inoculated with 800 ml. of a bacterial suspension in the logarithmic phase of growth. The growth vessel used was a modified stainless-steel container of a commercial washing machine (Hunt, Rogers & Hughes, 1959). Growth was maintained at 30° under conditions of vigorous aeration, formic acid (90%, w/v; A.R.) being added to keep the pH at 7·5–8·5. After 42 hr. the bacteria (31 g. wet wt.) were harvested in a Sharples centrifuge and stored at −10°.

Preparation of cell-free extracts. The harvested cells (0·5–2·0 g. wet wt.) were suspended in 2 vol. of mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·8, containing about 200 μg. each of crystalline ribonuclease and deoxyribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks). Extracts were then prepared by crushing the frozen suspension in a Hughes press (Hughes, 1951), at −20°. The crushed material was homogenized with a glass homogenizer and the resulting extract was used for some experiments. More frequently, the supernatant fraction obtained from the homogenate by centrifuging at 15 000 *g* for 20 min. in a refrigerated angle-head centrifuge was used. This fraction always contained 12–20 mg. of protein/ml.

Experiments with radioactive fixation. The standard-assay mixture for measuring isotope incorporation into 3-phosphoglyceric acid (PGA) from [^{14}C]bicarbonate or [^{14}C]-formate contained: 5 μmoles of tris buffer, pH 7·8, 2·5 μ-moles of $MgCl_2$, 0·5 μmole of ribulose 1:5-diphosphate, 2·5 μmoles of reduced glutathione (GSH), 0·5–2·5 μmoles of tracer, 0·05 ml. of bacterial extract and water to a final volume of 0·35 ml. The reaction was allowed to continue for 15 min. at 30°.

Fixation under anaerobic conditions was studied in a type of micro-Thunberg tube (Fig. 1). The tubes were blown from 6 mm. glass tubing and the appropriate solutions introduced into the bulbs with a hypodermic syringe. The tubes were evacuated to a pressure of 14 mm. Hg with a water pump and refilled with O_2-free N_2, the procedure being repeated six times in succession. The tubes were finally evacuated and sealed with a coal gas–O_2 hand blowpipe. The solutions were mixed by tipping and incubation was at 30°. The reaction was terminated by plunging the tubes into liquid air, and by opening the tubes while still frozen, pipetting in 1 ml. of ethanol and transferring the tubes to a boiling-water bath for 15 sec. The contents were then taken out of the tubes and treated similarly to the products of the aerobic experiments.

Measurement of radioactivity and analysis of labelled compounds. Direct measurement of the total radioactivity incorporated by bacterial extracts into non-volatile compounds was made by stopping the enzymic reaction with 3 vol. of hot ethanol. Samples (up to 0·25 ml.) of the reaction mixture were then plated in a stream of warm air on to glass planchets mounted on a rotating turntable. The plates were irrigated with 0·2 ml. of formic acid (90%, w/v; A.R.), and dried and counted with an end-window, lead-shielded G.M. tube (General Electric Co. Ltd.)

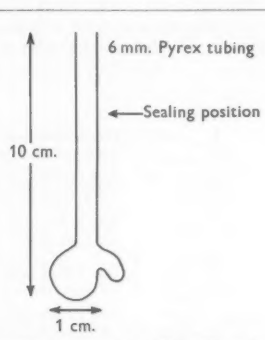


Fig. 1. Micro-Thunberg tube. After filling, the tubes were sealed off at the position indicated by the arrow.

* Part 1. Quayle & Keech (1959).

Samples of the ethanolic extracts of the reaction mixture were centrifuged and the residues washed with 0·2 ml. of hot aqueous 20 % (v/v) ethanol. The combined supernatant fractions were evaporated to dryness at 60° under N_2 and redissolved in aqueous 20 % (w/v) ethanol ready for application to the origin position of paper chromatograms. The resulting chromatographic analysis, radioautography and identification of labelled compounds followed the procedures previously described (Quayle & Keech, 1959). An independent measurement was made of the relation between the count of a radioactive sample on a glass planchet and on paper after chromatography. Under the conditions used, the planchet count was 1·5 times as high as the count obtained *in situ* after chromatography.

The formation of radioactive oxaloacetate in a reaction mixture was demonstrated by the addition of 5 μmoles of carrier oxaloacetate immediately before adding 0·5 ml. of 2:4-dinitrophenylhydrazine (0·1 % in 2 N-HCl). The mixture was incubated at 30° for 15 min. and the resulting hydrazone extracted into 2 ml. of ethyl acetate; it was extracted from the ethyl acetate with 1 ml. of 2 M-Na_2CO_3, acidified with HCl and re-extracted with 2 ml. of ethyl acetate. A sample of this solution was chromatographed in the solvent system of El Hawary & Thompson (1953).

Preparation of tracer solutions and manometric methods. The procedures used have been described previously (Quayle & Keech, 1959).

Chemicals. A sample of ribulose 1:5-diphosphate, used in the initial experiments, was a gift from Dr J. A. Bassham (Radiation Laboratory, University of California, Berkeley 4). The larger quantities subsequently used were prepared in this Laboratory from ribose 5-phosphate and adenosine triphosphate (ATP) (both chemicals from Schwarz Laboratories Inc., New York, U.S.A.), purified phosphoriboisomerase and phosphoribulokinase prepared from spinach according to the method of Horecker, Hurwitz & Weissbach (1956) being used. Glyceric acid was a gift from Dr P. W. Kent. Pyridine nucleotides were purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. Tetrahydrofolic acid was a gift from Dr R. Kisliuk. Sedoheptulose was a gift from Dr A. A. Benson (Department of Agricultural and Biological Chemistry, Pennsylvania State University, University Park, Pennsylvania).

Protein determinations. Protein was estimated by the method of Folin & Ciocalteu (1927).

RESULTS

Fixation of [^{14}C]*bicarbonate by the cell-free supernatant fraction*

Ribulose 1:5*-diphosphate-dependent carboxylation.* [^{14}C]Bicarbonate fixation into phosphorylated compounds by the cell-free supernatant fraction of extracts of *P. oxalaticus* was found to be dependent upon the addition of ribulose 1:5-diphosphate to the reaction mixture (Table 1). This activity was not removed from the extract by centrifuging for 30 min. at 100 000 *g* (Table 7). The requirement for ribulose 1:5-diphosphate as a substrate for the carboxylation reaction could be met by the addition of ribose 5-phosphate plus ATP to the reaction mixture (Table 2).

The observed fixation of [^{14}C]bicarbonate into ribulose 1:5-diphosphate was enhanced by the presence of $MgCl_2$ and GSH. Substitution of phosphate buffer for tris buffer reduced the amount of fixation to less than one-half of the previous activity. The carboxylating enzyme showed considerable sensitivity to the pH of the medium (Table 3), maximum incorporation being observed at pH 8·0. Increase in ribulose 1:5-diphosphate concentration beyond 1·4 mM, under the experimental conditions used, proved inhibitory, although at this concentration fixation was proportional to both the protein and [^{14}C]bicarbonate concentrations (Table 4). The rate of the ribulose 1:5-diphosphate-dependent carboxylation was linear over 15 min. (Fig. 2).

Table 1. *Effect of various factors on the fixation of* [^{14}C]*bicarbonate in the presence of ribulose* 1:5*-diphosphate*

The complete system contained 0·42 μmole of sodium bicarbonate (10 μc of ^{14}C), 0·05 ml. of *P. oxalaticus* extract (supernatant fraction), compounds added as indicated and water to 0·35 ml. Incubation was at room temperature for 15 min. and the reaction was stopped with 3 vol. of hot ethanol. Samples (0·1 ml.) were plated on glass planchets, and treated with formic acid, dried and the radioactivity was counted. Different bacterial extracts were used for Expts. 1 and 2.

Expt.	Additions	Total radioactivity fixed (counts/min.)
1	Tris buffer (pH 7·8; 5 μmoles) + ribulose 1:5-diphosphate (0·5 μmole) + $MgCl_2$ (2·5 μmoles) + GSH (2·5 μmoles)	5 770
	Tris buffer (pH 7·8; 5 μmoles) + ribulose 1:5-diphosphate (0·5 μmole) + $MgCl_2$ (2·5 μmoles)	4 090
	Tris buffer (pH 7·8; 5 μmoles) + $MgCl_2$ (2·5 μmoles) + GSH (2·5 μmoles)	20
2	Tris buffer (pH 7·8; 5 μmoles) + ribulose 1:5-diphosphate (0·5 μmole) + $MgCl_2$ (2·5 μmoles) + GSH (2·5 μmoles)	12 300
	Tris buffer (pH 7·8; 5 μmoles) + ribulose 1:5-diphosphate (0·5 μmole) + GSH (2·5 μmoles)	9 330
	Phosphate buffer (pH 7·8; 5 μmoles) + ribulose 1:5-diphosphate (0·5 μmole) + $MgCl_2$ (2·5 μmoles) + GSH (2·5 μmoles)	4 380

Table 2. *Fixation of* [^{14}C]*bicarbonate in the presence of ribose 5-phosphate and adenosine triphosphate*

The complete system contained 0·05 ml. of bacterial extract (supernatant), 5 μmoles of tris buffer (pH 7·8), 2·5 μmoles of $MgCl_2$, 2 μmoles of GSH, 1·25 μmoles of $NaH^{14}CO_3$ (containing 10 μc of ^{14}C), additions as indicated and water to 0·35 ml. Incubation was at 30° for 15 min. and the reaction was stopped with 3 vol. of hot ethanol. Resulting precipitates were washed with 0·2 ml. of ethanol (20%, v/v); half quantities of the resulting combined supernatants were chromatographed and the ratioactivity incorporated into the products was counted.

Additions	Total radioactivity fixed (counts/min.)
None	20
ATP (4 μmoles)	590
Ribose 5-phosphate (2 μmoles) + ATP (4 μmoles)	3280

Table 3. *Effect of* pH *on the fixation of* [^{14}C]*bicarbonate in the presence of ribulose* 1:5*-diphosphate*

The complete system contained 0·42 μmole of sodium bicarbonate (10 μc of ^{14}C), 0·05 ml. of *P. oxalaticus* extract (supernatant fraction; 25·9 mg. of protein/ml.), 5 μmoles of tris buffer, 0·5 μmole of ribulose 1:5-diphosphate, 2·5 μmoles of $MgCl_2$, 2·5 μmoles of GSH and water to 0·35 ml. Incubation was at room temperature for 15 min. and the reaction was stopped with 3 vol. of hot ethanol. The radioactivity in 0·1 ml. samples was assayed as described in Table 1.

pH	Total radioactivity fixed (counts/min.)
7·6	4360
7·8	7320
8·0	8070
8·2	6360
8·4	5920

Chromatographic analysis, followed by radioautography, of the products of the incubation showed that 80–90% of the radioactivity was present as PGA (Table 5). Alanine, aspartic acid, serine, malic acid and glyceric acid contained most of the remaining radioactivity.

No stimulation of ribulose 1:5-diphosphate-carboxylating activity in resin-treated extract (Dowex-1, chloride form; 200–400 mesh), by addition of diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) in either

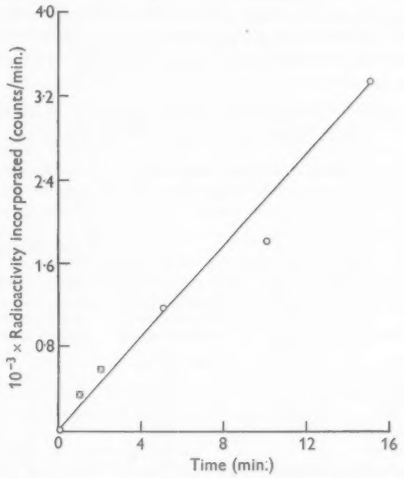


Fig. 2. Incorporation of [^{14}C]bicarbonate into PGA in the presence of ribulose 1:5-diphosphate. The incubation mixture was the same as that in Table 7, 0·45 μmole of $NaH^{14}CO_3$ (10 μc of ^{14}C) being used as tracer. Incubation and assay for radioactivity were as described in Table 1.

Table 4. *Dependence of fixation of* [^{14}C]*bicarbonate on ribulose* 1:5*-diphosphate, bicarbonate and enzyme concentrations*

The complete systems contained 5 μmoles of tris buffer (pH 7·8), 2·5 μmoles of $MgCl_2$, 2·5 μmoles of GSH, additions as indicated and water to 0·35 ml. The supernatant fraction of the *P. oxalaticus* extract contained 17·9 mg. of protein/ml. Incubation was at room temperature for 15 min. and treatment was as described in Table 1.

Expt.	Additions: Added to each tube	Additions: Variable addition		Total radioactivity fixed (counts/min.)
1	$NaH^{14}CO_3$ (0·4 μmole, 10 μc) + supernatant (0·05 ml.)	Ribulose 1:5-diphosphate	(0·2 μmole)	2440
			(0·5 μmole)	3130
			(1·0 μmole)	2170
2	Supernatant (0·05 ml.) + ribulose 1:5-diphosphate (0·5 μmole)	$NaH^{14}CO_3$	0·2 μmole, 5 μc	1460
			0·4 μmole, 10 μc	3130
			0·8 μmole, 20 μc	6241
3	Ribulose 1:5-diphosphate (0·5 μmole) + $NaH^{14}CO_3$ (0·4 μmole, 10 μc)	Supernatant	0·025 ml.	1307
			0·050 ml.	3130
			0·100 ml.	5200

oxidized or reduced forms, ATP, tetrahydrofolic acid, biotin, pyridoxal phosphate, coenzyme A or cyanocobalamin, singly or together, was observed.

The formation of PGA from [^{14}C]bicarbonate and ribulose 1:5-diphosphate in the extract shows the presence in this organism of an enzyme similar to the carboxydismutase found in photosynthetic tissue (Weissbach, Smyrniotis & Horecker, 1954; Quayle, Fuller, Benson & Calvin, 1954) and autotrophic bacteria (Santer & Vishniac, 1955; Trudinger, 1955). The increased incorporation of radioactivity observed in the presence of GSH and magnesium chloride, pH optimum and the inhibition observed in the presence of inorganic phosphate or excess of ribulose 1:5-diphosphate are all properties possessed by purified spinach carboxydismutase (Weissbach, Horecker & Hurwitz, 1956; Jakoby, Brummond & Ochoa, 1956).

Fixation of [^{14}C]*bicarbonate in the presence of pyruvate and phosphopyruvate.* The existence of a 'malic enzyme' (Ochoa, Mehler & Kornberg, 1948) catalysing the reductive carboxylation of pyruvate

$$(\text{pyruvate} + CO_2 + \text{TPNH} + H^+ \rightleftharpoons \text{malate} + \text{TPN}^+)$$

was suggested by the fixation of [^{14}C]bicarbonate into malic acid in the presence of pyruvate and either reduced DPN (DPNH) or reduced TPN (TPNH) (Table 6, Expt. 1); the latter coenzyme was more effective than the former. Malic acid contained over 90 % of the radioactivity fixed.

In the presence of phosphopyruvate incorporation of [^{14}C]bicarbonate into malate, citrate, aspartate and oxaloacetate (Table 6, Expts. 2 and 3) was observed. The appearance of malate and oxaloacetate as reaction products indicated the presence of a phosphopyruvate carboxylase (Bandurski & Greiner, 1953) catalysing the irreversible carboxylation of phosphopyruvate

$$(\text{phosphopyruvate} + CO_2 \rightarrow \text{oxaloacetate} + HPO_4^{2-})$$

or a nucleoside diphosphate-dependent oxaloacetate carboxylase or both (Utter & Kurahashi, 1954): phosphopyruvate + CO_2 + inosine diphosphate $\rightleftharpoons$ oxaloacetate + inosine triphosphate. The stimulation of fixation into oxaloacetate after the addition of inosine diphosphate (Table 6, Expt. 3) is evidence for the existence of the last-named enzyme.

Table 5. *Products of fixation of* [^{14}C]*bicarbonate in the presence of ribulose* 1:5*-diphosphate*

The incubation mixture was similar to that described in Table 3; 5 μmoles of tris buffer (pH 7·8) was used. The reaction products were analysed chromatographically as in Table 2.

Reaction product	Radioactivity incorporated (counts/min.)	Percentage of total non-volatile radioactivity incorporated into mixture
PGA	17 500	80
Aspartate	130	<1
Serine	880	4
Malate	820	4
Glycerate	353	2
Alanine	1428	6·5
Unknown compounds	670	3

Table 6. *Fixation of* [^{14}C]*bicarbonate in the presence of pyruvate and phosphopyruvate*

Micromanometer cups, flushed with $N_2 + CO_2$ (95:5), were used. The main compartments contained 20 μmoles of tris buffer (pH 7·4), 3 μmoles of $MgCl_2$, 5 μmoles of GSH, 11 μmoles of $NaH^{14}CO_3$ (20 μc) and additions as indicated; the side arms contained 0·05 ml. of bacterial extract (homogenate) and water to bring total volume in the flasks to 0·7 ml. Flasks were tipped after equilibration for 2 min. and incubated at 30° for 45 min. In Expts. 1 and 2 the reaction was stopped by adding 2 ml. of hot ethanol; the resulting precipitates were washed with ethanol (20 %, v/v), and the contents of the combined supernatants were chromatographed and the products assayed for radioactivity as described in Table 2. In Expt. 3, 5 μmoles of oxaloacetate was added before stopping the reaction with acid 2:4-dinitrophenylhydrazine solution. Resulting hydrazones were chromatographed and assayed for radioactivity as described above.

		Radioactivity (counts/min.)			
Expt.	Additions	Malate	Citrate	Aspartate	Oxaloacetate 2:4-dinitrophenylhydrazone
1	Pyruvate (4 μmoles)	0	0	0	—
	Pyruvate (4 μmoles) + DPNH (10 μmoles)	72	0	0	—
	Pyruvate (4 μmoles) + TPNH (10 μmoles)	1340	0	0	—
2	None	0	0	0	—
	Phosphopyruvate (5 μmoles)	5700	1000	2200	—
	Phosphopyruvate (5 μmoles) + inosine diphosphate (3·5 μmoles)	4050	720	2390	—
3	None	—	—	—	0
	Phosphopyruvate (5 μmoles)	—	—	—	1430
	Phosphopyruvate (5 μmoles) + inosine diphosphate (3·5 μmoles)	—	—	—	3190

The fact that citrate and aspartate appeared in Expt. 2 and not in Expt. 1 indicates that, under the experimental conditions, i.e. with DPNH or TPNH added, no appreciable amounts of oxaloacetate are formed.

Fixation of [^{14}C]formate in the presence of ribulose 1:5-diphosphate

Fixation of formate carbon into phosphorylated compounds by the cell-free supernatant fraction, as with [^{14}C]bicarbonate fixation, was found to be dependent on the addition of ribulose 1:5-diphosphate (Table 7). Analysis of the reaction products by chromatography and radioautography revealed a pattern of labelled compounds similar to that obtained with [^{14}C]bicarbonate as tracer; 80–90 % of the radioactivity appeared in PGA. No stimulation of this activity was observed in resin-treated extract (Dowex 1, chloride form; 200–400 mesh) by addition of the same cofactors as were tested in the carboxylation reaction.

It was important to know whether this incorporation of formate carbon depended upon either a prior transformation of the formate to carbon dioxide (followed by a carboxylation of ribulose 1:5-diphosphate, as demonstrated in the previous experiments) or the formation of a C_1 unit, different from carbon dioxide, which was involved in PGA production from ribulose 1:5-diphosphate. The following experiments were performed to distinguish between these possibilities.

Removal of [^{14}C]formate-fixing activity by centrifuging. The ability to incorporate formate carbon was completely removed by centrifuging the extract at 100 000 *g* for 30 min. (Table 7). Recombination of the particulate fraction (14 000–100 000 *g*) with the supernatant fraction restored the capacity of the system to incorporate [^{14}C]formate to 65 % of the original value in the whole extract. The particulate fraction possessed the ability to incorporate carbon both from [^{14}C]formate and [^{14}C]bicarbonate in the presence of ribulose 1:5-diphosphate (Table 8). However, the incorporation from both substrates was reduced by over 75 % after washing it twice in small volumes of tris buffer. Recombination of the washed particles with the supernatant fraction restored the [^{14}C]formate-fixing activity.

Oxidation of formate by the particulate fraction. The cell-free extract rapidly oxidized formate, and in a Warburg manometer 98 % of the theoretical oxygen uptake was observed (Table 9). After

Table 7. *Separation by centrifuging of* [^{14}C] *formate- and* [^{14}C]*bicarbonate-fixing activities*

The reaction mixture contained 5 μmoles of tris buffer (pH 7·8), 2·5 μmoles of $MgCl_2$, 2·5 μmoles of GSH, 0·05 ml. of extract, 0·2 μmole of ribulose 1:5-diphosphate, tracer as indicated and water to 0·35 ml. Incubation was at 30° for 15 min. Radioactivity incorporated was assayed as described in Table 1.

Omissions	Centrifugal interval (10^{-3} × relative centrifugal force) (*g*)	Fraction	Total radioactivity (counts/min.) incorporated/mg. of protein: $NaH^{14}CO_3$ (1·2 μmoles, 5 μc)	Total radioactivity (counts/min.) incorporated/mg. of protein: $H^{14}CO_2Na$ (1·2 μmoles, 5 μc)
None	—	Whole extract	4000	6400
Ribulose 1:5-diphosphate	—	Whole extract	330	300
None	0–14	Supernatant	5270	2140
None	14–100	Supernatant	5650	380
Ribulose 1:5-diphosphate	14–100	Supernatant	—	310
None	14–100	Supernatant	—	4200
	14–100	+ Particulate		

Table 8. *Effect of washing on the* [^{14}C]*formate- and* [^{14}C]*bicarbonate-fixing activities of the particulate fraction*

Particulate fraction obtained from 0·8 g. (wet wt.) of bacteria was disrupted in a Hughes press and homogenized in 2 ml. of mM-tris buffer (pH 7·8). The centrifugal fraction 1600–10 000 *g* for 15 min. was resuspended in 0·7 ml. of tris buffer to give fraction R_1; this procedure was repeated twice in succession to give R_2 and R_3 respectively. Fractions were tested for enzymic activity as described in Table 7.

Omissions	Fraction	Total radioactivity (counts/min.) incorporated in assay mixture: $NaH^{14}CO_3$ (1·2 μmoles, 5 μc)	Total radioactivity (counts/min.) incorporated in assay mixture: $H^{14}CO_2Na$ (1·2 μmoles, 5 μc)
None	R_1	2600	1280
None	R_2	540	1010
None	R_3	430	175
Ribulose 1:5-diphosphate	R_2	55	110

centrifuging, all the oxidizing activity was found in the particulate fraction, whereas the supernatant fraction was devoid of such activity.

These experiments demonstrate the existence in the extract of a system capable of ribulose 1:5-diphosphate-dependent incorporation of formate: an oxidation of the formate to carbon dioxide catalysed by the particulate fraction, followed by carboxylation of ribulose 1:5-diphosphate by the supernatant fraction. The experiments do not, however, show if this pathway was responsible for all the observed [^{14}C]formate fixation, or whether, by the concerted action of supernatant and particulate fractions, some C_1 unit other than carbon dioxide was involved.

It has been shown that hypophosphite, an inhibitor of formic dehydrogenase in some organisms (Takamiya, 1953), stops the oxidation of formate by washed suspensions of *P. oxalaticus* at a concentration of mM (Quayle & Keech, 1959). Carboxydismutase is not inhibited by this concentration of hypophosphite and hence the study of the effect of hypophosphite on formate incorporation into PGA in the cell-free extract seemed an obvious approach to the problem. It was found, however, that formate oxidation, as well as formate incorporation into PGA, by the cell-free extract was completely unaffected by the presence of mM-hypophosphite. The non-inhibition by hypophosphite of formate oxidation in the extracts was an unexpected finding, since with other organisms the compound functions as an inhibitor of formate oxidation both in whole cells and cell-free extracts (Campbell, 1954, 1955; H. L. Kornberg & A. M. Gotto, personal communication), and requires further investigation. The alternative approach of studying [^{14}C]formate incorporation under anaerobic conditions was then tried.

Anaerobic fixation of [^{14}C]*formate.* The experiments were performed in micro-Thunberg tubes filled with nitrogen at a pressure of about 15 mm. Hg. Under these conditions the incorporation into PGA was reduced to one-third of the value obtained under aerobic conditions (Table 10). The addition of methylene blue to the system increased the anaerobic incorporation of radioactive carbon into PGA to 72% of the aerobic incorporation. Radioactive malate was formed under all conditions.

Table 9. *Oxidation of formate by the cell-free extract*

Particulate fraction was obtained from the bacterial extract by centrifuging (0–30 000 *g* for 15 min.). Manometer cups contained: main compartment, whole extract (12 mg. dry wt.), or particulate fraction (11 mg. dry wt.), in 71 mM-sodium phosphate (pH 7·0); side arm, substrate; centre well, 400 μmoles of KOH. Total volume of contents was 3 ml. Incubation was at 30° for 1 hr.

Fraction	Formate (μmoles)	O_2 uptake (μl.)	Theoretical O_2 uptake (μl.)
Whole extract	None	20	—
Whole extract	20	218	224
Particulate	20	213	224
Supernatant	20	13	224

DISCUSSION

Evidence for the operation of a carbon dioxide-fixation cycle in *P. oxalaticus* (OX 1) grown on formate, similar to that found in photosynthetic and other autotrophic organisms, has been presented in the preceding paper. Confirmation of this is provided in the present work by demonstration of the presence of the two enzymes peculiar to such a cycle, namely phosphoribulokinase and carboxydismutase, in the cell-free bacterial extracts. Phosphoriboisomerase is also present. This enzyme is not peculiar only to the Calvin cycle of carbon dioxide fixation, but is also involved in reactions of the pentose phosphate cycle.

The participation of a C_1 unit other than carbon dioxide in the observed formation of PGA from ribulose 1:5-diphosphate and formate in the

Table 10. *Anaerobic fixation of* [^{14}C]*formate by the extract*

The supernatant fraction obtained after centrifuging at 1600 *g* for 10 min. was used in the following reaction mixture: 5 μmoles of tris buffer (pH 7·8), 2·5 μmoles of $MgCl_2$, 2·5 μmoles of GSH, 0·05 ml. of extract, 2·5 μmoles of $H^{14}CO_2Na$ (10 μc) and water to 0·35 ml. Anaerobic tests were carried out in sealed micro-Thunberg tubes filled with N_2 under reduced pressure. Incubation was at 30° for 15 min. Reaction was stopped with 1 ml. of hot ethanol and the products were chromatographed and the radioactivity was counted as described in Table 2.

Additions	Experimental conditions	Radioactivity (counts/min.) incorporated: PGA	Malate
None	Aerobic	0	0
None	Anaerobic	0	0
Ribulose 1:5-diphosphate (0·5 μmole)	Aerobic	1570	511
Ribulose 1:5-diphosphate (0·5 μmole)	Anaerobic	570	108
Ribulose 1:5-diphosphate (0·5 μmole) + methylene blue (0·3 μmole)	Anaerobic	1250	190

extracts is discounted on the basis of the following facts:

(*a*) The formate-fixing activity of the extract disappears on centrifugal removal of a particulate fraction which is capable of oxidizing formate to carbon dioxide.

(*b*) PGA formation from formate and ribulose 1:5-diphosphate is greatly reduced under anaerobic conditions, when formate cannot be oxidized to carbon dioxide.

The fact that PGA production was not completely arrested anaerobically is considered to be an experimental limitation rather than evidence for some incorporation of formate itself. The presence of a formic dehydrogenase in the crude extract could lead to anaerobic dismutation of formate to carbon dioxide and formation of a reduced coenzyme. Re-oxidation of this coenzyme by an electron acceptor could then lead to a continuous oxidation of formate. The formation of radioactive malate under anaerobic conditions is evidence for one such system operating in the extracts. The synthesis of malate by reductive carboxylation of pyruvate, or carboxylation of phosphopyruvate followed by reduction of the resulting oxaloacetate, would accomplish the re-oxidation of a reduced pyridine nucleotide; such enzymes were present in the extract.

The presence of these carboxylating enzymes in the cell could also explain the observation (Quayle & Keech, 1959) that radioactive carbon from [^{14}C]bicarbonate or [^{14}C]formate was rapidly incorporated into malate by growing cells.

SUMMARY

1. Reactions of [^{14}C]formate and [^{14}C]bicarbonate in cell-free extracts (prepared with a Hughes press) of *Pseudomonas oxalaticus* (OX 1) grown on formate have been studied.

2. The extracts catalyse the incorporation of [^{14}C]bicarbonate into 3-phosphoglycerate in the presence of either ribulose 1:5-diphosphate or ribose 5-phosphate plus adenosine triphosphate. These findings show the existence of phosphoribo-isomerase, phosphoribulokinase and carboxydismutase in the extracts.

3. 3-Phospho[^{14}C]glyceric acid is also formed in the extracts from [^{14}C]formate and ribulose 1:5-diphosphate. This enzymic activity is removed by centrifuging the extract and restored by recombining the particulate and supernatant fractions.

4. Incubation under anaerobic conditions reduced the phosphoglycerate formation from [^{14}C]-formate to one-third of that observed on aerobic incubation. Addition of methylene blue restored the anaerobic incorporation to 72% of that observed under aerobic conditions.

5. The particulate fraction of the extract oxidizes formate to carbon dioxide.

6. These results show that the ribulose 1:5-diphosphate-dependent incorporation of [^{14}C]-formate in the extract is preceded by oxidation of the formate to carbon dioxide. The small amount of formate incorporation still observed under anaerobic conditions is considered to be due to experimental limitation and not to some incorporation of formate itself.

7. The extracts incorporate [^{14}C]bicarbonate into malate in the presence of pyruvate and reduced di- or tri-phosphopyridine nucleotide. The latter coenzyme was the more effective. [^{14}C]-Bicarbonate incorporation into malate and oxaloacetate in the presence of phosphopyruvate is catalysed by the extracts.

We wish to thank Professor Sir Hans A. Krebs, F.R.S., for his interest and encouragement, and Mrs M. Jenkins for her skilled technical assistance. This work was supported by the Rockefeller Foundation and by the Office of Scientific Research of the Air Research and Development Command of the United States Air Force, through its European Office, under contract no. AF. 61(514)-1180, and was done while one of us (D. B. K.) held an Overseas Scholarship of the Commonwealth Scientific and Industrial Research Organization (Australia).

REFERENCES

Bandurski, R. S. & Greiner, C. M. (1953). *J. biol. Chem.* **204**, 781.

Bassham, J. A., Benson, A. A., Kay, L. D., Harris, A. Z., Wilson, A. T. & Calvin, M. (1954). *J. Amer. chem. Soc.* **76**, 1760.

Campbell, L. L. (1954). *J. Bact.* **68**, 598.

Campbell, L. L. (1955). *J. biol. Chem.* **217**, 669.

El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.

Folin, O. & Ciocaltéu, V. (1927). *J. biol. Chem.* **73**, 627.

Horecker, B. L., Hurwitz, J. & Weissbach, A. (1956). *J. biol. Chem.* **218**, 785.

Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.

Hunt, A. L., Rogers, A. & Hughes, D. E. (1959). *Biochim. biophys. Acta* (in the Press).

Jakoby, W. B., Brummond, D. O. & Ochoa, S. (1956). *J. biol. Chem.* **218**, 811.

Ochoa, S., Mehler, A. H. & Kornberg, A. (1948). *J. biol. Chem.* **174**, 979.

Quayle, J. R., Fuller, R. C., Benson, A. A. & Calvin, M. (1954). *J. Amer. chem. Soc.* **76**, 3610.

Quayle, J. R. & Keech, D. B. (1958). *Biochim. biophys. Acta*, **29**, 223.

Quayle, J. R. & Keech, D. B. (1959). *Biochem. J.* **72**, 623.

Santer, M. & Vishniac, W. (1955). *Biochim. biophys. Acta*, **18**, 157.

Takamiya, A. (1953). *J. Biochem., Tokyo*, **40**, 415.

Trudinger, P. A. (1955). *Biochim. biophys. Acta*, **18**, 581.

Utter, M. F. & Kurahashi, K. (1954). *J. biol. Chem.* **207**, 787.

Weissbach, A., Horecker, B. L. & Hurwitz, J. (1956). *J. biol. Chem.* **218**, 795.

Weissbach, A., Smyrniotis, P. Z. & Horecker, B. L. (1954). *J. Amer. chem. Soc.* **76**, 3611.

Ion Transport and Metabolism in Slices of Guinea-Pig Seminal-Vesicle Mucosa

By R. WHITTAM and H. J. BREUER
Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford, and Chemische Abteilung, Chirurgische Universitäts-Klinik, Bonn, Germany

(*Received* 5 *January* 1959)

Lardy & Phillips (1943), and Blackshaw (1953) found that bull and ram spermatozoa show optimum motility *in vitro* only when at least 5–10 mM-K^+ ions are present in the medium. Potassium also stimulates the metabolism of spermatozoa, and Dott & Walton (1956) have shown that both glycolysis and respiration are needed for maximum motility. Potassium is therefore essential for the motility and metabolism of spermatozoa. It is present in the natural milieu for spermatozoa on ejaculation, namely seminal plasma, which is elaborated mainly by the seminal vesicles in the rat, guinea pig and bull (see Mann, 1954). As a preliminary to the study of the secretion of ions in seminal plasma, Breuer & Whittam (1957) tried to find out how the high potassium concentration was maintained in slices of seminal-vesicle mucosa of the guinea pig. They showed that anaerobiosis had little effect on the tissue sodium and potassium concentrations during incubation at 37°, but that inhibitors of glycolysis anaerobically or of respiration aerobically caused a loss of potassium and a gain of sodium. Metabolic energy from either respiration or anaerobic glycolysis was therefore needed to support the concentration gradients of sodium and potassium, which they suggested may depend upon a supply of adenosine triphosphate, the formation of which is common to both metabolic pathways. The electrical potential difference across the cell membranes is not known and it is not possible therefore to decide whether both potassium and sodium are actively transported. Thus the intracellular potassium concentration could be maintained either by a primary active transport of potassium or by a primary active outward movement of sodium, which would generate a potential difference and cause a passive inward movement of potassium. The present work has shown that, whether sodium or potassium or both ions are actively transported, there is a correlation between the retention of potassium and the exclusion of sodium, and the concentration of easily hydrolysable phosphate. Other experiments are described which show that a reduction in the sodium concentration of the medium caused a roughly proportional fall in the tissue sodium concentration, without the tissue potassium concentration being affected. Under these conditions the intracellular potassium is probably retained passively by non-diffusible anions.

METHODS

Materials and procedure. Guinea pigs weighing 400–600 g. were stunned and killed by bleeding from the carotid artery, and the seminal vesicles removed immediately. Slices of seminal-vesicle mucosa were made with a dry razor blade as described by Breuer & Whittam (1957). The slices of mucosa (30–50 mg.) were then placed in 3 ml. of medium (described below) in 15 ml. stoppered conical cups, which were shaken at 1°, 17°, 27° or 37° in a water bath. In anaerobic experiments, a stick of yellow phosphorus was placed in the centre well. After various periods, the slices were taken from the cups, blotted on Whatman no. 541 filter paper and weighed.

Media. The phosphate medium of Krebs & Henseleit (1932) was used as the standard medium. Low-sodium and sodium-free media were prepared by partially or completely substituting 0·154 M-choline chloride or 0·308 M-sucrose for the NaCl, and 10 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris) chloride buffer, pH 7·4, for the phosphate buffer. Unless otherwise stated, glucose (20 mM) and sodium L-glutamate (10 mM) were added to the media as substrates. Glutamate was added because Breuer & Whittam (1957) found that a higher potassium and a lower sodium concentration were maintained in the tissue in its presence.

Determination of sodium and potassium. The weighed tissue slices were placed in 5 ml. of N-HNO_3 and allowed to stand overnight (to leach the soluble salts from the tissue). Samples (1 ml.) of the acid extract of the tissue were diluted to 5 ml. for the flame-photometric estimation of Na and K with the flame-photometer attachment of the Beckman DU spectrophotometer.

Determination of chloride. Chloride was determined, on a 1 ml. sample of the HNO_3 extract of the tissue, by the potentiometric-titration method of Sanderson (1952).

Determination of easily hydrolysable phosphate. Mucosal slices (30–50 mg.) were placed in 1 ml. of 5% (w/v) trichloroacetic acid, broken up with a glass rod and allowed to stand at 4° during the night. The samples were then shaken and centrifuged. Samples (0·4 ml.) of the supernatant acid extract were taken for the estimation of inorganic phosphate by the method of Berenblum & Chain (1936). For the estimation of easily hydrolysable phosphate, 1 ml. of N-HCl was added to 0·2 ml. of the extract in

trichloroacetic acid, and the mixture heated for 7 min. in a boiling-water bath and the inorganic phosphate then estimated. The value for easily hydrolysable phosphate was obtained by subtraction of the inorganic phosphate concentration before acid hydrolysis from that found after heating for 7 min. at 100° in N-HCl. Easily hydrolysable phosphate is taken to originate mainly from adenosine triphosphate (ATP) and creatine phosphate.

Measurement of respiration and anaerobic glycolysis. The consumption of O_2 and liberation of CO_2 by the mucosa was measured in Warburg manometers at 1°, 17°, 27° and 37° in the usual way. The results are expressed as Q_{O_2} and Q_{CO_2} (μl./mg. dry wt./hr.).

Water content of tissue. The tissue water was taken to be equal to the loss of weight on drying the tissue overnight at 105°.

RESULTS

Concentrations of potassium and easily hydrolysable phosphate in tissue slices

Previous experiments showed that anaerobiosis did not prevent the retention of potassium and the exclusion of sodium by slices of seminal-vesicle mucosa, although cooling and inhibitors of respiration and glycolysis did prevent this (Breuer & Whittam, 1957). A maintenance of concentration gradients in the same tissue, under both aerobic and anaerobic conditions, eliminates any mechanism of cation transport depending on consumption of O_2 and electron transport in the cytochrome system. An alternative possibility is that easily hydrolysable phosphate (e.g. ATP or creatine phosphate) might support active cation transport, both aerobically and anaerobically, by energizing a carrier mechanism. In this case, low and high potassium concentrations would be expected with low and high concentrations of easily hydrolysable phosphate respectively, irrespective of whether potassium is actively transported inwards directly or follows as a consequence of active sodium transport outwards. This expectation has been tested by measuring the concentrations of easily hydrolysable phosphate in slices containing either low or high concentrations of potassium. These were obtained by incubation at 1° or 37°, and in the presence or absence of metabolic inhibitors.

To obtain enough tissue for incubation under the six conditions shown in Table 1 the tissue from two animals was required. Slices were immersed in the standard medium at room temperature until they had all been made (about 10 min.), and then transferred to the conical cups for gassing and incubation. Analysis of the tissue immediately after slicing showed that the potassium concentration fell by 21 μmoles/g. of whole tissue, and that the sodium concentration rose by a similar amount, during the immersion in the medium (Table 1). The reason for these changes is not clear, but they may be partly due to a loss of potassium and a gain of sodium by damaged cells on the outside of the slices. This explanation requires that about 18 % of the cells would be damaged. It is also suggested by the similar loss of potassium that occurred after immersion in 0·308 M-sucrose solution (see Table 4). This loss was then about the same after 2 min. (22 μmoles/g.) as after 45 min. (27 μmoles/g.), as would be expected from an equilibration of damaged cells with the bathing solution. For this reason, the concentrations found after incubation have been compared with the values for the tissue after immersion in the medium at room temperature and not with those for freshly prepared slices.

Table 1. *Concentrations of easily hydrolysable phosphate and of potassium and sodium in slices of seminal-vesicle mucosa after incubation* in vitro *for 45 min.*

20 mM-Glucose and 10 mM-sodium glutamate were added to the medium, pH 7·4. Mean values of at least two results are given. When added, the concentration of 2:4-dinitrophenol (DNP) was 0·2 mM and that of sodium iodoacetate (IAA) was 1 mM.

			Concentrations (μmoles/g. of whole tissue)				Change during incubation (μmoles/g. of whole tissue)		
			Easily hydrolysable phosphate	K	Na	Na + K	Easily hydrolysable phosphate	K	Na
Tissue after dry-slicing			—	109	35	144	—	—	—
Tissue before incubation			8·9	88	67	155	—	—	—
Tissue after incubation									
Temp.	Gas phase	Inhibitors added							
37°	O_2	None	8·1	84	75	159	−0·8	−4	+8
37	N_2	None	8·8	75	85	160	−0·1	−13	+18
37	O_2	DNP + IAA	5·4	48	108	156	−3·5	−40	+41
37	N_2	IAA	4·7	50	104	154	−4·2	−38	+37
1	O_2	None	5·9	39	113	152	−3·0	−49	+46
1	N_2	None	5·7	35	115	150	−3·2	−53	+48

The results of incubation for 45 min. at 37° show that the concentration of easily hydrolysable phosphate (8·9 μmoles/g.) remained approximately constant under both aerobic (8·1 μmoles/g.) and anaerobic (8·8 μmoles/g.) conditions (Table 1). After aerobic incubation, the tissue potassium concentration was practically unchanged (84 μmoles/g.), whereas anaerobically it fell 15%, from 88 to 75 μmoles/g. The addition of 0·2 mM-2:4-dinitrophenol plus mM-sodium iodoacetate aerobically and of iodoacetate anaerobically caused respective losses of 39 and 47% in the easily hydrolysable phosphate concentration, and of 45 and 42% in the potassium concentration. Under these conditions, the sodium concentration increased by an amount approximately equivalent to the loss of potassium. After either aerobic or anaerobic incubation at 1°, the loss of easily hydrolysable phosphate was 35% and that of potassium about 60%, and, again, the loss of potassium was balanced by an equivalent gain of sodium. The percentage losses of easily hydrolysable phosphate and of potassium at 1° are therefore only roughly correlated, possibly because cooling retarded the rate of hydrolysis of phosphate esters without markedly changing the rate of loss of potassium. The absence of major changes in the concentrations of easily hydrolysable phosphate and of sodium and potassium under both aerobic and anaerobic conditions at 37°, and the comparable losses of easily hydrolysable phosphate and potassium, and the gains of sodium, produced by metabolic inhibitors, suggest that the retention of potassium and the exclusion of sodium are probably facilitated by labile phosphate esters.

Effect of temperature on the tissue ionic concentration

To find whether a reduction of the temperature of incubation would cause movements of tissue sodium, potassium and chloride to levels which might be correlated with the metabolic energy supplies, as shown by the Q_{O_2} and $Q^{N_2}_{CO_2}$ values, slices were incubated at 37°, 27°, 17° and 1° under both aerobic and anaerobic conditions. The results are shown in Table 2. The sum of the concentrations of sodium plus potassium was approximately constant at about 160 μmoles/g., irrespective of the temperature of incubation, which shows that any losses of potassium were balanced by equivalent amounts of sodium. The Q_{O_2} and $Q^{N_2}_{CO_2}$ values were each about 7 after incubation at 37°, and fell by 52 and 34% respectively when the temperature was reduced from 37° to 27°. The ionic concentrations changed by only 7–14% on reducing the temperature from 37° to 27°, and are not therefore directly related to the metabolic quotients. The energy available from metabolism at 27° was probably sufficient to meet the demands of the active transport processes. At 17°, the Q_{O_2} and $Q^{N_2}_{CO_2}$ values were approximately only 20 and 30% of those at 37°. The reduced energy supply then probably limited the active movements of ions, and accounts for the lower concentration of potassium (58·5–61·0 μmoles/g.), and the higher concentrations of sodium (103–106 μmoles/g.) and chloride (68·0–74·5 μmoles/g.) found aerobically and anaerobically. At 1°, the loss of tissue potassium and the gains of sodium and chloride were even greater than at 17°, and the O_2 consumption and CO_2 output were immeasurably small with the Warburg apparatus.

The intracellular concentrations of sodium and potassium have also been calculated, a value of 13 ml./100 g. of tissue being used to correct for the ions in the extracellular space. This was the inulin space previously found under both aerobic and anaerobic conditions at 37° (Breuer & Whittam, 1957), and it has been assumed also to represent the extracellular space at the lower temperatures. The intracellular potassium concentrations are likely to be accurate since the correction for extracellular potassium was small. The sodium concentration, on the other hand, can be considered as only approximate in view of the large correction for extracellular sodium and the likelihood of small variations in the inulin space. Fig. 1 shows the results of aerobic incubation and Fig. 2 those of anaerobic incubation. Both graphs show that whereas the concentrations depended on the temperature of

Table 2. *Effect of the temperature of incubation on the potassium, sodium and chloride concentrations, and the metabolic quotients of seminal-vesicle mucosa*

Slices of mucosa (about 30–60 mg.) were shaken for 1 hr. in 3 ml. of phosphate or bicarbonate medium containing 20 mM-glucose and 10 mM-glutamate, pH 7·4.

Gas phase		O_2				N_2+CO_2 (95:5)			
Temp.		37°	27°	17°	1°	37°	27°	17°	1°
Q_{O_2} or $Q^{N_2}_{CO_2}$		6·7	3·2	1·4	<0·2	7·3	4·8	2·4	<0·2
Ionic concentrations (μmoles/g. of whole tissue)	K^+	87·0	79·5	58·5	40·0	74·5	69·0	61·0	35·0
	Na^+	69·0	79·0	106·0	113·5	85·0	92·0	103·0	115·0
	Cl^-	61·5	66·0	74·5	—	44·5	52·0	68·0	—
	$Na^+ + K^+$	158·0	158·5	164·5	153·5	159·5	161·0	164·0	150·0

incubation they were not simply related to the rates of respiration or of anaerobic glycolysis. A lower potassium concentration, at temperatures below 37°, was accompanied by a higher sodium concentration, and the sum of the two concentrations was approximately constant (148–165 μmoles/g. of cells). A similar dependence of chloride concentrations on temperature was shown in slices of guinea-pig-kidney cortex by Whittam (1956), without a simple relationship between concentration and metabolic rate.

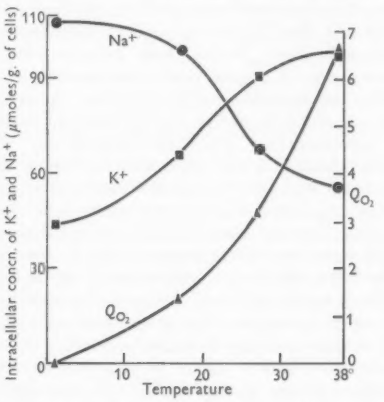


Fig. 1. Effect of the temperature of incubation on the intracellular sodium and potassium concentrations and the Q_{O_2} of seminal-vesicle mucosa. Slices were incubated for 45 min. in phosphate standard medium.

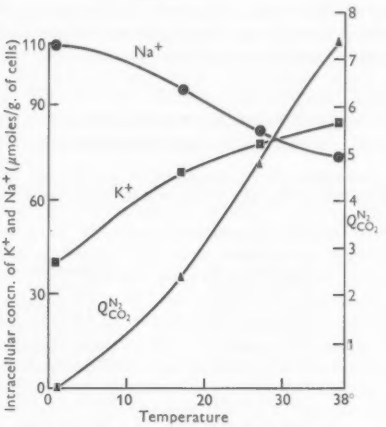


Fig. 2. Effect of the temperature of incubation on the intracellular sodium and potassium concentrations and the $Q^{N_2}_{CO_2}$ of seminal-vesicle mucosa. Slices were incubated for 45 min. in bicarbonate standard medium.

Effects of lowering the sodium concentration of the medium

Tissue sodium concentration. The concentration gradient of sodium between cells and medium in a sodium-free solution, made isotonic with either a non-penetrating electrolyte or non-electrolyte, will favour a movement of sodium from cells to medium instead of from medium into cells as in the medium of Krebs & Henseleit (1932). The potassium concentration of the tissue in the absence of external penetrating cations may be due to a Donnan equilibrium or to potassium movements either directly or indirectly dependent upon metabolism. To find the effect of the sodium concentration of the medium on the tissue sodium and potassium concentrations, slices of seminal-vesicle mucosa have been incubated aerobically at 37° in media in which sodium chloride had been partially or completely replaced with choline chloride or sucrose. Choline choloride and sucrose were chosen as replacements for sodium chloride as examples of a relatively non-penetrating electrolyte and non-electrolyte. Although the extent of their penetration into slices of seminal-vesicle mucosa was not determined, it seemed unlikely from results with other cells that this would be appreciable during incubation times of 45 min.

The results showed that the tissue sodium concentration was proportional to that of the medium over the range 20–120 mM, and that it was constant (about 65 μmoles/g. of whole tissue) when the medium contained 120 and 155 mM-Na^+ ions (Fig. 3). In sodium-free solution, the tissue sodium concentration was not significantly different from zero. Similar results were found after incubation in

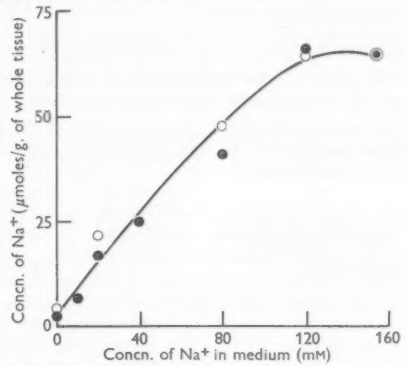


Fig. 3. Effect of sodium concentration in the medium on the sodium concentration of slices of seminal-vesicle mucosa after aerobic incubation for 45 min. at 37°. ●, Medium containing sucrose; ○, medium containing choline chloride.

the sucrose or choline chloride media. The results in Fig. 3 show that the cell membranes were permeable to Na^+ ions, which migrated from cells to medium in the low-sodium and sodium-free solutions. The lower tissue concentrations found at higher external sodium concentrations (120 and 155 mM) must have been due to the active extrusion of sodium from the cells.

To find the rate at which tissue sodium leaked into sodium-free media, the tissue was analysed for sodium after incubation for various times. Fig. 4 shows that the tissue sodium concentration fell to the same extent in both the choline chloride and sucrose media. This fall represents a loss of sodium from extra- and intra-cellular fluid, although a clear resolution into two components is not possible from the curve. The graph shows, however, a rapid initial fall gradually merging into a slower fall, as would be expected from a more rapid loss from extracellular fluid than from within the cells.

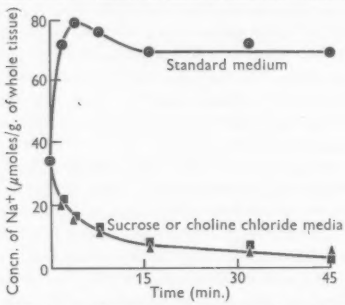


Fig. 4. Effect on the sodium concentration in slices of seminal-vesicle mucosa of aerobic incubation at 37° in standard medium (●) or in sodium-free sucrose (■) or choline chloride (▲) media.

Fig. 4 also shows that after about 30–45 min., the tissue sodium had become experimentally indistinguishable from zero. In contrast with the loss of tissue sodium in sodium-free media, during incubation in the medium containing 160 mM-Na^+ ion, the tissue sodium concentration increased almost twofold in the first 2 min. and then stayed approximately constant (70 μmoles/g. of whole tissue) for the remainder of the 45 min. incubation (Fig. 4).

Tissue potassium concentration. Results in Table 3 show that a partial or complete replacement of sodium with sucrose in the medium had no significant effect on the potassium concentration of incubated slices, which was between 81 and 86 μmoles/g. of whole tissue irrespective of the sodium concentration of the medium. After incubation in the sodium-free choline chloride medium, the concentration was 72·5 μmoles/g. of whole tissue, which is lower than that found in tissue from the standard medium. The difference of 13 μmoles/g. (85·5 − 72·5), with S.E. 2·8, is derived from mean values from a number of experiments in which there was some scatter, and is significant ($P < 0.01$). It is probably due to an entry of choline into the cells in exchange for potassium, for Maizels & Remington (1958) have recently shown that choline partially displaces potassium in renal-cortex slices, and it cannot be regarded as a completely non-penetrating cation. No attempt has been made to identify the anion which must leave the cells with sodium during incubation in sodium-free sucrose medium although chloride seems the most likely. In choline chloride medium, a loss of intracellular chloride is most unlikely, however, in view of the high external concentration, and sufficient choline may penetrate to balance the loss of sodium and the small loss of potassium.

Table 3. *Effect of the replacement of sodium chloride in the medium with either sucrose or choline chloride on the potassium concentration, the water content and Q_{O_2} of slices of seminal-vesicle mucosa*

About 30–60 mg. of tissue was shaken for 45 min. at 37° in 3 ml. of medium, pH 7·4, containing 20 mM-glucose, 10 mM-glutamate and 5 mM-K^+ ion. Gas phase was O_2. Mean values and S.E. are given, and the figures in parentheses are the number of observations.

Compound with which NaCl was replaced	Concn. of sodium in medium (mM)	Q_{O_2}	Concn. of potassium in tissue (μmoles/g. of whole tissue)	Water content (%, w/w)
Choline chloride	0	4·0±0·4 (5)	72·5±2·1 (20)	79·1±0·7 (12)
	5	—	71·5±1·1 (6)	79·0±0·1 (10)
	20	—	76·0±1·8 (8)	80·2±0·3 (12)
	40	—	75·0±1·8 (8)	79·8±0·1 (12)
	80	5·9±0·4 (6)	81·5±1·2 (8)	80·4±0·3 (12)
	120	—	78·5±0·9 (14)	80·7±0·4 (10)
Sucrose	0	3·7±0·2 (10)	81·0±1·4 (8)	79·3±1·0 (6)
	20	—	85·0±0·8 (6)	79·4±0·2 (8)
	80	5·2±0·2 (9)	83·5±1·6 (8)	79·5±0·5 (8)
	120	—	83·0±0·9 (8)	81·2±0·1 (8)
Control in standard medium	155	6·4±0·1 (36)	85·5±1·9 (16)	80·5±0·4 (10)

Table 4. *Sodium and potassium concentrations in slices of seminal-vesicle mucosa after incubation at 37° in standard medium or in sodium-free media*

Slices of tissue (about 50 mg.) were incubated in media, pH 7·4, containing 10 mM-tris buffer, 10 mM-glutamate, 20 mM-glucose and 5 mM-K^+ ion. Gas phase was O_2. Mean values are given ± S.E. and the figures in parentheses are the number of observations. Abnormal media are named after the principal solute.

Medium ...	Concn. of potassium (μmoles/g. of whole tissue)		
	Standard	Choline chloride	Sucrose
Time of incubation (min.)			
0	108·7 ± 1·5 (15)	108·7 ± 1·5 (15)	108·7 ± 1·5 (15)
2	81·9 ± 1·8 (6)	85·6 ± 3·7 (8)	86·7 ± 2·0 (10)
4	77·2 ± 2·1 (6)	85·4 ± 1·7 (8)	81·7 ± 2·5 (10)
8	81·7 ± 4·0 (6)	79·2 ± 2·9 (8)	83·5 ± 2·7 (10)
16	86·7 ± 3·4 (8)	75·5 ± 1·0 (8)	81·4 ± 3·0 (6)
32	87·8 ± 4·0 (12)	72·4 ± 1·5 (8)	81 3 ± 3·2 (6)
45	83·9 ± 1·6 (28)	72·5 ± 2·1 (20)	81·2 ± 1·4 (8)

After incubation in either the standard medium or in sodium-free media, the tissue lost potassium (about 20–30 μmoles/g. of whole tissue) in the first 2–4 min. (Table 4). This loss occurred in both phosphate and bicarbonate media, and is probably due to a loss of potassium from damaged cells on the outside of the slice. Table 4 shows that the tissue potassium concentration was not significantly different after incubation in the sodium-free sucrose medium and in the standard medium (81·2 and 83·9 μmoles/g. of whole tissue), although it was significantly lower in choline chloride medium (72·5 μmoles/g. of whole tissue) (see also Table 3).

Rate of respiration. The rate of respiration of the tissue in standard medium at 37° of 6·4 μl./mg. dry wt./hr. was reduced by 19% by a lowering of sodium concentration of the medium to 80 mM (Q_{O_2} 5·2–5·9), and by 42% in the sodium-free media (Q_{O_2} 3·7–4·0). Significant differences were not found between the choline chloride and sucrose media. [The tissue-water content (79–81%) was not changed by incubation in the low-sodium media (Table 3).]

Effect of metabolic inhibitors. The addition of 2:4-dinitrophenol to slices of seminal-vesicle mucosa respiring in standard medium caused a loss of potassium, which was balanced by an uptake of sodium, and this was part of the evidence indicating a dependence of ionic concentrations on metabolism (Breuer & Whittam, 1957). The question now arises whether the potassium concentration found after incubation in sodium-free media is also dependent on metabolism, whether directly or indirectly, or whether it is due to a Donnan effect arising from the presence of non-diffusible anions. Fig. 4 shows that, after incubation in sodium-free, sucrose or choline chloride media, the tissue sodium concentration approached zero, and Table 4 that the potassium concentration was approximately equal to that after incubation in standard medium. If retention of potassium in the cells in sodium-free media is a result of a Donnan equilibrium, interference with metabolism should not affect it, but if it is due to active transport, then metabolic inhibitors would be expected to lower the concentration.

Slices of mucosa have therefore been incubated in the standard medium, and in the sodium-free sucrose and choline chloride media, in the presence of 2:4-dinitrophenol and iodoacetate, which were added to inhibit both respiration and glycolysis. The inhibitors added to the standard medium caused a lowering of the potassium concentration by 24 μmoles/g. of whole tissue anaerobically, and by 28 μmoles/g. of whole tissue aerobically, compared with the control slices, which contained about 75 μmoles/g. of whole tissue (Table 5). In contrast, the inhibitors caused only a small loss of tissue potassium (4–13 μmoles/g. of whole tissue) in the sucrose or choline choloride media, both aerobically and anaerobically. The media contained 10 mM-glucose and 10 mM-L-glutamate, and it seemed possible that this small loss might be due to a movement of glutamate accompanied by potassium.

Effect of glucose and glutamate. To test this possibility, slices were incubated aerobically in each medium without the addition of glucose and glutamate. The potassium concentration was then 70 μmoles/g. of whole tissue after incubation in the sucrose medium, and 57 μmoles/g. of whole tissue after incubation in the choline chloride medium (Table 5). The values are 13 μmoles/g. lower than those found after incubation with glucose and glutamate. These effects are almost identical with those found after the addition of 2:4-dinitrophenol plus iodoacetate in the presence of glucose and

glutamate, and show that about 10–13 μmoles of potassium/g. of whole tissue, of the concentration found after incubation in sodium-free media containing glucose and glutamate, was due to the presence of these compounds and to the availability of metabolic energy. The results are compatible with an active uptake of 10–13 μmoles/g. of glutamate accompanied by potassium, which was abolished by 2:4-dinitrophenol plus iodoacetate.

In contrast with the small effects of glucose and glutamate on the tissue potassium concentrations in sodium-free media, a large difference of 31 μmoles of potassium/g. of whole tissue was found in tissue incubated in the standard medium, with and without glucose and glutamate (Table 5). Again, this difference is similar to that found by adding 2:4-dinitrophenol plus iodoacetate (28 μmoles/g.). If an active movement of glutamate accompanied by potassium occurs to the same extent as in the tissue incubated in the sodium-free media, it would explain only about 40 % of the effects in the standard medium. The greater effects in tissue incubated in the standard medium are undoubtedly due to the presence of external sodium, which was able to replace internal potassium in metabolically deficient tissues.

Effect of cooling. To test further whether a high tissue potassium concentration could be maintained in the absence of metabolism, slices were incubated at 1°, since cooling should cause a loss of potassium maintained by a coupling to metabolism, but not when the potassium concentration is due to a Donnan equilibrium. Table 5 shows that the tissue potassium concentration was 40 μmoles/g. of whole tissue after incubation at 1° in standard medium, whereas that from sucrose medium was 76 μmoles/g. of whole tissue, and that from choline chloride medium 56 μmoles/g. of whole tissue.

The results in Table 5 therefore show that a high tissue potassium concentration could be maintained during incubation in sucrose or choline chloride media in the virtual absence of metabolic energy. In standard medium, only a low tissue potassium concentration was then found since the intracellular potassium could be readily replaced by external Na^+ ions (see Table 1). In the absence of external Na^+ ions, owing to a replacement with sucrose or choline, internal potassium was therefore retained passively, probably by a Donnan equilibrium.

DISCUSSION

Easily hydrolysable phosphate and potassium and sodium concentrations

The main result of the incubation of slices of seminal-vesicle mucosa of the guinea pig in medium at 37° is that, under both aerobic and anaerobic conditions, the concentrations of easily hydrolysable phosphate (taken to be mainly ATP) and of potassium underwent only small changes. The easily hydrolysable phosphate concentration would not be expected to be as high anaerobically as aerobically, because the rates of oxygen uptake and carbon dioxide output were the same, with Q_{O_2} and $Q^{N_2}_{CO_2}$ values about 7. Now, by assuming a phosphorylation quotient (P/O ratio) of about 3, it can be shown that for the production of ATP a Q_{O_2} of 1 is approximately equivalent to a $Q^{N_2}_{CO_2}$ of 6 (see Warburg, 1956). It follows therefore that the $Q^{N_2}_{CO_2}$ was only about one-sixth of that required to

Table 5. *Effect of cooling, the addition of glucose and glutamate and the addition of 2:4-dinitrophenol plus iodoacetate on the potassium concentration of seminal-vesicle mucosa after incubation in standard medium or in sodium-free sucrose or choline chloride media*

About 30–40 mg. of tissue was shaken for 45 min. in 4 ml. of solution in 15 ml. conical flasks containing 10 mM-tris buffer, pH 7·4. Concentrations were glucose, 20 mM; L-glutamic acid, 10 mM; 2:4-dinitrophenol (DNP), 0·2 mM and iodoacetate (IAA), 1 mM.

Medium	Gas phase	Temp.	Concn. of potassium (μmoles/g. of whole tissue): With glucose + glutamate: No DNP + IAA (*a*)	With glucose + glutamate: With DNP + IAA (*b*)	No glucose and glutamate (*c*)	Loss of potassium (μmoles/g. of whole tissue): DNP + IAA [(*a*) − (*b*)]	Glucose + glutamate omitted [(*a*) − (*c*)]	Cooling
Standard	O_2	37°	76	48	45	28	31	36
	O_2	1	40	—	—	—	—	
	N_2	37	74	50	41	24	33	—
Sucrose	O_2	37	83	72	70	11	13	7
	O_2	1	76	—	—	—	—	
	N_2	37	76	72	—	4	—	—
Choline chloride	O_2	37	70	60	57	10	13	14
	O_2	1	56	—	—	—	—	
	N_2	37	73	60	—	13	—	—

produce ATP at the same rate anaerobically as aerobically. The similar concentrations found under these conditions suggest that, for the synthesis to keep pace with the loss of ATP, the rate of hydrolysis anaerobically must be but one-sixth of the rate aerobically.

An increase in the efflux of sodium from squid giant axons poisoned with cyanide, on the injection of ATP (Caldwell & Keynes, 1957; Keynes, 1958; Caldwell, 1958), is the most direct evidence for a linkage of ATP with the active transport of a cation (sodium), and a little less so are the parallel falls in ATP concentration and potassium influx in glucose-free, human erythrocytes (Whittam, 1958). These are examples of cells which are respectively predominantly aerobic and exclusively anaerobic in their metabolism. The seminal-vesicle mucosa, on the other hand, maintains its potassium by energy from either metabolic pathway and, in this respect, resembles frog skin, which can actively transport sodium under anaerobic conditions at about 60–80 % of the rate under aerobic conditions (Leaf & Renshaw, 1957). It seems likely that ATP provided by either respiration or glycolysis is utilized by the ion-transport mechanism in these different cells and tissues.

Whatever unknown factors control the rate of hydrolysis of ATP, the energy available from anaerobic glycolysis was sufficient to support a potassium concentration almost as high anaerobically as aerobically. Losses of easily hydrolysable phosphate and of potassium, and a gain of sodium, occurred when metabolic inhibitors (2:4-dinitrophenol plus iodoacetate) were added at 37°, and on cooling to 1°. The maintenance of the concentrations of easily hydrolysable phosphate and of sodium and potassium both aerobically and anaerobically at 37°, and the rough parallelism between the net losses of potassium and labile phosphate, suggest that the active transport of sodium, and possibly of potassium, probably depends on labile phosphate esters. These results do not distinguish between whether both sodium and potassium are subject to primary active-transport processes or whether potassium is distributed passively as a consequence of active sodium transport. In either case, however, labile phosphate esters seem to be associated with the primary active process.

Under the conditions of incubation when the concentration of labile phosphate fell, the possibility has to be considered that the orthophosphate produced might leave the cells accompanied by potassium and thus account for the fall in potassium concentration. This possibility can be discounted because the loss of potassium was from three to ten times greater than could be accounted for by a loss of monobasic orthophosphate accompanied by potassium. (The discrepancy would be even greater if account is taken of the mixture of mono- and di-basic orthophosphate more likely to be present.) The loss of potassium and gain of sodium therefore seem to be due to a failure of active transport, which is correlated with hydrolysis of labile phosphate.

Tissues seem to fall into three classes from the way they depend on metabolic energy for the active transport of ions. First, there are those tissues and cells which are predominantly aerobic in their metabolism, and which require respiratory energy for the active transport of ions. Examples are chicken erythrocytes (Maizels, 1954), rat diaphragm (Creese, 1954) and skeletal muscle (McLennan, 1955), guinea-pig brain and kidney cortex, retina (Krebs, Eggleston & Terner, 1951; Mudge, 1951; Whittam & Davies, 1953) and squid giant axons (Hodgkin & Keynes, 1955). Secondly, cells which can transport ions anaerobically and do not possess a respiratory pathway are human erythrocytes (Harris, 1941; Maizels, 1949). Thirdly, frog skin (Leaf & Renshaw, 1957), seminal-vesicle mucosa (Breuer & Whittam, 1957), duck erythrocytes (Tosteson & Johnson, 1957) and ascites tumour cells (Maizels, Remington & Truscoe, 1958) possess the ability to transport cations actively both aerobically and anaerobically, thus combining in the same cells the features of the first two types.

Potassium retention in the absence of medium in the sodium

The finding that the replacement of sodium chloride in the medium with either sucrose or choline chloride caused almost a complete loss of tissue sodium shows that the cells in slices of seminal-vesicle mucosa are permeable to sodium. The retention of tissue potassium during incubation in the sodium-free media was largely unaffected by cooling, the addition of metabolic inhibitors and the deprivation of substrates (glucose and glutamate), although these factors caused a large loss of potassium from tissue incubated in standard medium. This result suggests that non-diffusible, intracellular anions set up a Donnan equilibrium, which probably accounts for the high tissue potassium found after incubation in the sodium-free sucrose and choline chloride media.

SUMMARY

1. Conditions of incubation are described under which slices of seminal-vesicle mucosa of the guinea pig retained most of their potassium in the absence of oxygen and of sodium in the medium. A comparison has been made between the ionic concentrations, the concentrations of easily hydrolysable phosphate and the rates of respiration and anaerobic glycolysis.

2. The tissue maintained its initial concentration of easily hydrolysable phosphate, and retained most of its potassium during both aerobic and anaerobic incubation for 45 min. at 37° in standard medium containing sodium chloride. The addition of 2:4-dinitrophenol and iodoacetate or a reduction of the temperature caused a loss of easily hydrolysable phosphate and of potassium, and a gain of sodium.

3. The rough correlation between the maintenance of the concentrations of sodium and potassium, and of easily hydrolysable phosphate, is compatible with a dependence of active cation transport on labile phosphate esters such as adenosine triphosphate.

4. Lowering the temperature of incubation from 37° to 27° did not affect the concentrations of sodium, potassium and chloride found in the tissue after both aerobic and anaerobic incubation, although Q_{O_2} and $Q^{N_2}_{CO_2}$ were reduced by about 50%. Incubation at 17° and 1°, however, caused a loss of potassium, gains of sodium and chloride and a further fall in the metabolic quotients. It appears that the energy supply was sufficient for the active transport of ions at 27° but not at 17° and 0°.

5. Tissue slices incubated in media in which sodium chloride had been partially or completely replaced by either choline chloride or sucrose contained sodium in a concentration roughly proportional to that of the medium. Their potassium concentration, however, remained unchanged in sucrose medium and fell only about 10 μmoles/g. of whole tissue in choline chloride medium. Incubation in sodium-free media caused a fall in Q_{O_2} from 6·4 in standard medium to about 4 in the sucrose or choline chloride media.

6. The tissue potassium concentration after incubation in sodium-free media was lowered only between 4 and 14 μmoles/g. of whole tissue by a reduction of temperature from 37° to 1°, or an addition of 2:4-dinitrophenol plus iodoacetate or an omission of glucose and glutamate (which were usually added to the medium). In contrast, these factors caused a large loss of potassium (24–36 μmoles/g. of whole tissue), which was balanced by a gain of sodium, after incubation in standard medium.

7. The results suggest that potassium can be retained passively, by a Donnan equilibrium created by non-diffusible internal anions, in slices of seminal-vesicle mucosa in the absence of metabolic energy, provided that sodium in the medium is replaced by sucrose or choline chloride. Potassium retention by the tissue in standard medium, however, depended on metabolic energy, because in its absence potassium was replaced by sodium.

We wish to thank Professor Sir Hans Krebs, F.R.S., for his helpful comments on the manuscript. The work was supported by grants from the Rockefeller Foundation (in Oxford) and the Deutsche Forschungsgemeinschaft (in Bonn).

REFERENCES

Berenblum, I. & Chain, E. (1936). *Biochem. J.* **32**, 295.
Blackshaw, A. W. (1953). *J. Physiol.* **120**, 46S.
Breuer, H. J. & Whittam, R. (1957). *J. Physiol.* **135**, 213.
Caldwell, P. C. (1958). *Proc. 4th Int. Congr. Biochem., Vienna.*
Caldwell, P. C. & Keynes, R. D. (1957). *J. Physiol.* **137**, 12*P*.
Creese, R. (1954). *Proc. Roy. Soc.* B, **142**, 497.
Dott, H. M. & Walton, A. (1956). *J. Physiol.* **133**, 30*P*.
Harris, J. E. (1941). *J. biol. Chem.* **141**, 579.
Hodgkin, A. L. & Keynes, R. D. (1955). *J. Physiol.* **128**, 28.
Keynes, R. D. (1958). *Proc. 4th Int. Congr. Biochem., Vienna.*
Krebs, H. A., Eggleston, L. V. & Terner, C. (1951). *Biochem. J.* **48**, 530.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Lardy, H. A. & Phillips, P. H. (1943). *Amer. J. Physiol.* **138**, 741.
Leaf, A. & Renshaw, A. (1957). *Biochem. J.* **65**, 90.
McLennan, H. (1955). *Biochim. biophys. Acta*, **24**, 1.
Maizels, M. (1949). *J. Physiol.* **108**, 247.
Maizels, M. (1954). *J. Physiol.* **125**, 263.
Maizels, M. & Remington, M. (1958). *J. Physiol.* **143**, 27S.
Maizels, M., Remington, M. & Truscoe, R. (1958). *J. Physiol.* **140**, 80.
Mann, T. (1954). *The Biochemistry of Semen.* London: Methuen.
Mudge, G. H. (1951). *Amer. J. Physiol.* **165**, 113.
Sanderson, P. H. (1952). *Biochem. J.* **52**, 502.
Tosteson, D. C. & Johnson, J. (1957). *J. cell. comp. Physiol.* **50**, 169.
Warburg, O. (1956). *Science*, **123**, 309.
Whittam, R. (1956). *J. Physiol.* **131**, 542.
Whittam, R. (1958). *J. Physiol.* **140**, 479.
Whittam, R. & Davies, R. E. (1953). *Biochem. J.* **55**, 880.

The Products of the Partial Acid Hydrolysis of the Mucopeptide from Cell Walls of *Micrococcus lysodeikticus*

By H. R. PERKINS and H. J. ROGERS
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 3 *February* 1959)

Salton & Horne (1951) and McCarty (1952*a*, *b*) isolated insoluble material from disintegrated Gram-positive bacterial cells, which was shown by electron-microscopic examination to have retained the form of the cells from which it had been isolated, and both sets of authors concluded that they had isolated the cell walls. The procedure involved disintegrating the cells by shaking a thick suspension with fine glass beads, separating the insoluble walls from unbroken cells and soluble cell contents by differential centrifuging and then repeatedly washing the wall material with water or sodium chloride solution. Enzyme preparations which would lyse the living organisms also lysed cell walls (McCarty, 1952*a*; Salton, 1952, 1956).

Subsequently a somewhat different method was described (Cummins & Harris, 1956) for preparing wall material. This method replaced the repeated washing procedure of Salton & Horne (1951) by treatment with the enzymes trypsin, ribonuclease and pepsin. Qualitative chemical examination (Salton, 1953; Cummins & Harris, 1956) of acid hydrolysates of wall material isolated by either method showed that it had a rather simple composition. When obtained from most species it yielded four or five amino acids, some in the D-configuration (Ikawa & Snell, 1956; Snell, Radin & Ikawa, 1955), one or two hexoses and two hexosamines. It is proposed to call the material mucopeptide. The two hexosamines usually present are glucosamine and its 3-*O*-α-carboxyethyl derivative. The latter was first recognized as an unknown amino sugar universally present in mucopeptide by Cummins & Harris (1956), and was more exactly characterized by Strange & Dark (1956); it has since been synthesized by Kent (1958) (see also Strange & Kent, 1959).

Although the earlier authors thought the material we propose to call mucopeptide constituted the whole cell wall of the organism, later work has somewhat complicated this picture. For example, Baddiley, Buchanan & Carss (1958) and Armstrong, Baddiley, Buchanan & Carss (1958) have shown that a polyribitol phosphate with combined amino acids in it can be extracted from cell-wall preparations from some species. The exact inter-relationship of the mucopeptide and these other substances is not clear. It seems likely, however, that the mucopeptide does not represent the whole cell wall in some species. Nevertheless, observations such as those of Park (1958) and Mandelstam & Rogers (1959) show that antibiotics such as penicillin and bacitracin inhibit its synthesis, and other strongly suggestive observations (Park & Johnson, 1949; Park, 1952*a*, *b*; Park & Strominger, 1957) have pointed to the importance of the inhibition of mucopeptide synthesis as a mechanism of action of some antibiotics.

Apart from investigations of the action of antibiotics, the mucopeptides themselves have great intrinsic interest in that they represent examples of substances containing only a limited number of both sugars and amino acids, yet, apparently, bearing considerable immunological specificity (Hundley, Ing & Krauss, 1956). Elucidation of their chemistry may help towards a further understanding of the more complicated mucoproteins.

A preliminary account of some of this work has already appeared (Perkins & Rogers, 1958).

MATERIALS AND METHODS

Growth of organisms and preparation of mucopeptide

Micrococcus lysodeikticus (National Collection of Type Cultures no. 2665) was grown on the surface of Hedley Wright (Wright, 1933) nutrient agar in Roux bottles for 48 hr. at 35°. To obtain large yields of organisms it was necessary to dry the agar thoroughly before inoculation. At the end of the period of incubation the cells were scraped from the surface of the medium with a glass rod, suspended in water (approx. 100 ml. for cells from 20 Roux bottles), washed twice with an equal volume of water and finally suspended in water to give a density of 20 mg. dry wt./ml. This suspension in 10 ml. volumes was mixed with an equal volume of glass beads and shaken on a Mickle (1948) disintegrator for 15 min. at 0–4°. The beads were removed by filtering the suspension through a Grade 1 sintered-glass filter and washed. The combined filtrate and washings were then centrifuged at 1100 *g* for 10 min. to sediment the unbroken cells. Some mucopeptide is also sedimented during this process, but it can be separated from the whole cells by carefully scraping the pellet with a spatula, the mucopeptide being distinguished from the whole cells by its white colour; the whole cells are bright yellow. The supernatant from this centrifuging was then

centrifuged again at 11 000 *g* for 10 min., during which the mucopeptide was sedimented. The deposit was washed twice with water and treated with trypsin, ribonuclease and pepsin as described by Cummins & Harris (1956). Finally the mucopeptide was washed twice with water and dried from the frozen state. Before analysis the preparation was heated to constant weight at 105°.

Analytical methods

Paper chromatography. Descending chromatography on Whatman no. 3 paper was used throughout the work. The solvent systems found most useful were butanol–acetic acid–water (6·3:1·0:2·7, by vol.) and butanol–pyridine–water (6:4:3, by vol.; Jeanes, Wise & Dimler, 1951); for the latter it is essential that the pyridine should be freshly redistilled. The papers were subsequently sprayed with 0·1 % ninhydrin in butanol for the qualitative examination of amino acids. For sugars, either the silver nitrate method of Trevelyan, Procter & Harrison (1950) was used, or the aniline phthalate spray of Partridge (1949). The amino sugar spots were made visible with the acetyl acetone and *p*-dimethylaminobenzaldehyde reagents of Partridge (1948).

Purification of paper. When substances were to be eluted from paper for subsequent analysis it was essential to use paper which had been washed. Whatman no. 3 paper initially containing low levels of peptide material was washed in N-HCl and then extensively with water until free of Cl^- ions. By this procedure the amount of elutable peptide in most batches of paper was reduced to insignificant quantities. Some batches required more vigorous treatment, such as that described by Hanes, Hird & Isherwood (1952).

Paper electrophoresis. The apparatus described by Kunkel (1954) was used with a potential difference of 700 v, usually applied for 3–4 hr. The following volatile buffers were found most useful: (1) N-acetic acid, pH 2·37; (2) triethylamine carbonate, pH 8·0 (Porath, 1956); (3) collidine–acetic acid (Newton & Abraham, 1954).

Estimation of amino acids. The method of Kay, Harris & Entenman (1956) was as modified by Mandelstam & Rogers (1959).

Estimation of hexosamines. In preliminary investigations the hexosamines liberated from the cell walls were estimated together as glucosamine by the method of Elson & Morgan (1933). *N*-Acetyl amino sugars were estimated by the method of Reissig, Strominger & Leloir (1955). Estimations of glucosamine and muramic acid separately were made as described below.

Separation of hexosamines. After hydrolysis of the samples in 4N-HCl for 4 hr. at 105° the glucosamine and muramic acid were separated by the method of J. T. Park (1957, personal communication). A column (5 mm. in diameter) of a mixture of Norit A (0·25 g.) and Celite (Johns-Manville Co. Ltd., London) (coarse, acid-washed, 0·25 g.) was washed with 2N-HCl. The hydrolysate was diluted to 2N with respect to HCl and a sample equivalent to about 4 μmoles of total hexosamine (measured as glucosamine) was added to the column. Elution with 10 ml. of water yielded the glucosamine and a further 10 ml. of 5 % (v/v) ethanol in water eluted the muramic acid. That separation of the two amino sugars was complete was proved in the first experiments by paper-chromatographic examination of the eluates. The hexosamines were estimated in samples of suitable volume by the method of Rondle & Morgan (1955), recrystallized glucosamine hydrochloride being used as a standard. The concentration of muramic acid was calculated on the assumption that, under the above-mentioned conditions, 1 mg. of muramic acid produced 27 % of the colour at 530 mμ given by 1 mg. of the free base, glucosamine (Crumpton, 1958).

Terminal amino groups in peptide. Peptides dissolved in dilute $NaHCO_3$ solutions were treated with an ethanolic solution of fluorodinitrobenzene (Sanger, 1945) for 2 hr. at room temperature. After removal of the excess of reagent by extraction with benzene the product was hydrolysed in 4N-HCl for 4 hr., at which time the destruction of dinitrophenyl amino acids, even dinitrophenyl glycine, is slight (Porter, 1957). The dinitrophenyl amino acids were separated by two-dimensional paper chromatography with (*a*) the buffered *tert.*-amyl alcohol (2-methylbutan-2-ol) solvent of Blackburn & Lowther (1951) and (*b*) the phosphate buffer of Levy (1954). All five dinitrophenyl amino acids and the two amino sugar derivatives could be separated by this means.

α-Amino nitrogen. This was estimated by the method of Yemm & Cocking (1955).

Glucose. This was estimated by first passing the neutralized hydrolysates (equivalent to 5–10 mg. of cell wall), obtained by heating for 2 hr. with 2N-H_2SO_4 at 100° (these conditions gave maximum liberation of reducing sugar), through a column (8·5 cm. × 1·1 cm.) of Zeo-Karb 225 resin (H^+ form). The column was then washed with water (20 ml.). During this process the amino sugars and amino acids, which otherwise also react in reducing-sugar tests, are retained on the column. Examination of the effluent and washings from a column, to which cell-wall hydrolysate had been applied, by paper chromatography showed that only glucose was present. Reducing sugar in the effluent and washings was estimated by the method of King (1947). *N*-Acetylglucosamine was present in certain fractions and this substance is also not retained by the resin; its amount was estimated independently and its reducing equivalent subtracted from the total value determined.

Total phosphorus. This was estimated by the modification of the Briggs method described by Allen (1940).

Incubation with lysozyme. Samples were incubated with a final concentration of 50 μg. of crystalline lysozyme/ml. (Armour Laboratories Ltd., Eastbourne, Sussex) in 0·1M-ammonium acetate buffer, pH 6·1, at 37° for periods up to 20 hr.

Incubation with carboxypeptidase. Samples of some of the fragments obtained by partial hydrolysis of mucopeptide were incubated with carboxypeptidase (Armour Laboratories Ltd.) essentially as described by Fraenkel-Conrat, Harris & Levy (1955), and the digest was examined quantitatively for amino acids by paper chromatography. The number of moles of enzyme used was either one-fiftieth or one-tenth of the number of moles of glycine present in the sample studied, and incubation was carried out for various periods either at room temperature or at 37°.

RESULTS

Quantitative analysis of the cell wall

Comparatively little quantitative knowledge about the mucopeptide of Gram-positive cell walls exists and, in view of our eventual aim of understanding the structure, it was necessary that more

Table 1. *Quantitative analysis of the cell walls of* Micrococcus lysodeikticus

Two separate batches (C.W. 18 and C.W. 20) of cell wall were analysed. All results are expressed as the basis of wall dried to constant weight at 105°. Optical rotations of the amino acids were not determined.

	C.W. 18		C.W. 20	
	%	Molar ratio*	%	Molar ratio*
Alanine	20·2	2·5	16·3	1·9
Glycine	10·8	1·6	8·5	1·2
Lysine	13·1	1·0	13·3	0·95
Glutamic acid	13·0	1·0	14·1	1·0
Glucosamine	10·1	0·64	12·8	0·75
(As *N*-acetylglucosamine)	12·4	—	15·8	—
Muramic acid	19·8	0·89	22·0	0·92
(As *N*-acetylmuramic acid)	23·1	—	25·7	—
Glucose	4·1	0·31	4·0	0·28

* Expressed in relation to glutamic acid.

precise information should be obtained. Table 1 shows two sets of results for mucopeptide samples. In both instances about 97 % of the dry matter can be accounted for in terms of the five amino acids, two *N*-acetylated amino sugars and glucose. In other batches analysed the total accounted for was never less than 94 %. This calculation assumes that all the glucosamine and muramic acid present are in the *N*-acetylated form: it does not, however, take account of the *O*-acetyl which is present (Abrams, 1958; Brumfitt, Wardlaw & Park, 1958). The difference between this total result and 100 % is within the experimental error and suggests that if other substances, not estimated, are present their concentration must be low. Despite the relative agreement between the values for the total weight accounted for, discrepancies between the values for the individual components fall outside the experimental error. The sets of results shown in Table 1 were selected from a number of analyses to show the greatest extent of this variation. For example, the results for alanine differ by 25 % and those for glycine by 20–30 %. These differences are similar to those obtained during the examination of batches of mucopeptide from *Staphylococcus aureus* (Mandelstam & Rogers, 1959). Such results may suggest the possible heterogeneity of the mucopeptides.

Measurement of the total phosphorus content of the wall mucopeptide gave a result of 0·1 %, which would correspond to a polyribitol phosphate content of about 0·7 %.

Course of acid hydrolysis of the mucopeptide

In the first experiments mucopeptide (7 mg.) was mixed with hot (100°, 2 ml.) 2N-H_2SO_4 and heated in a boiling-water bath. At the times indicated in Figs. 1 and 2 samples were withdrawn, cooled rapidly to 0–4° and neutralized with solid $BaCO_3$. The precipitated $BaSO_4$ and any excess of $BaCO_3$ were removed by centrifuging, washed with an equal volume of water and the combined washings and supernatant fluid analysed. It can be seen from Fig. 1 that glucose was rapidly liberated, about 70 % appearing after 5 min. and the remainder after a further 40–50 min. Material in the hydrolysate which reacted to give colour in the *N*-acetylhexosamine test rose to a maximum in 20 min. and then rapidly declined. Not all this material was free *N*-acetylhexosamine, however, since when the hydrolysate was passed through a Zeo-Karb 225 resin (H^+ form) column, about 20 % of it was retained. This material could be eluted with 0·3N-HCl (Fig. 1). As the hydrolysis proceeded the amount of hexosamine-reacting substance, as distinct from the *N*-acetylated sugar, increased rapidly for the first hour and then remained approximately constant during the second hour of treatment. When the amount of hexosamine which was retained by the column but eluted by 0·3N-HCl was measured it rose steadily throughout the hydrolysis period of 2 hr. Some, however, which behaved very differently in its rate of elution by 0·3N-HCl, was clearly not free amino sugar. This material is studied in detail below. Fig. 2 shows the rate of liberation of free amino nitrogen.

Since the purpose was to study compounds containing both amino acids and amino sugars, it seemed reasonable to choose a time of hydrolysis which would leave the maximum amount of hexosamine or *N*-acetylhexosamine in some form different from the free amino sugars. The difference in behaviour on ion-exchange columns seemed a reasonable criterion and at 20 min. the concentration of such compounds was at a maximum. At this time about 80 % of the glucose was present as free hexose but only about 15–20 % of the total amino nitrogen had been liberated.

Attempts to replace 2N-H_2SO_4 as a hydrolytic agent by other reagents were for various reasons unsuccessful. Among those tried were 2N-acetic acid, 0·1N-HCl, 0·1N-H_2SO_4 and N-NaOH, all at

100°, and 10N-HCl at 37°. Attention was therefore directed towards the 20 min. hydrolysate in which 2N-H_2SO_4 had been used.

Fractionation of the hydrolysate

The neutralized ($BaCO_3$) hydrolysates were dried from the frozen state and then treated as follows.

Dialysis. The dry material was dissolved in a small volume of water and dialysed three times with agitation against 10 vol. of water, each time overnight at 0–4°. Of the dry weight in the hydrolysate 95% was diffusible under these conditions and this diffusate was dried from the frozen state. The non-diffusible part was shown to contain all the components of the cell wall but was not examined further.

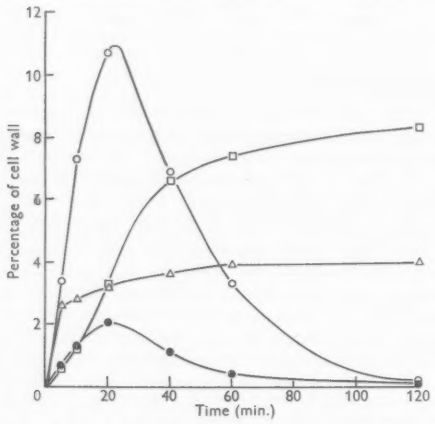


Fig. 1. Course of acid hydrolysis of mucopeptide with 2N-H_2SO_4 at 100°. ○, *N*-Acetylhexosamine expressed in terms of *N*-acetylglucosamine; □, hexosamine, and ●, bound hexosamine, both expressed in terms of glucosamine; △, glucose.

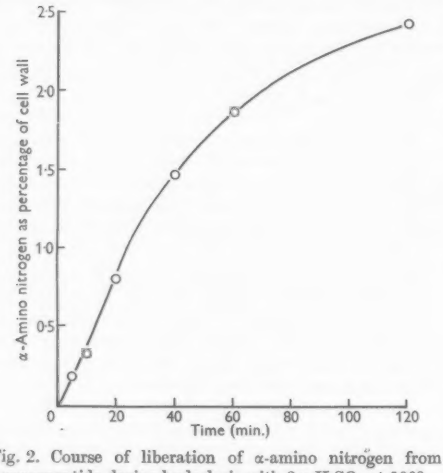


Fig. 2. Course of liberation of α-amino nitrogen from mucopeptide during hydrolysis with 2N-H_2SO_4 at 100°.

Column chromatography. A solution in water containing diffusible material from 500 mg. of mucopeptide was applied to a column (25 cm. × 1·3 cm.) of Dowex-50 (H^+ form, 8% cross-linked). Water was passed through the column and 2·3 ml. fractions were collected. Fractions 3–9 contained reducing material and were concentrated *in vacuo* before examination by paper chromatography. After about 70 ml. of water had passed through the column further material was eluted with 0·3N-HCl and the fractions were examined for *N*-acetylhexosamine and for hexosamine. A single peak was present in the fractions 16–30. The yield of dry material was 17 mg. The next peak eluted by 0·3N-HCl contained free glucosamine and muramic acid.

Paper chromatography in butanol–acetic acid–water. (*a*) Material not retained by Dowex-50. A solution of the material was applied as a broad band to the washed paper, which was irrigated with the solvent for 12 hr. When sprayed for amino sugars, *N*-acetylglucosamine and *N*-acetylmuramic acid were shown to be present, together with an intermediate band ($R_{glucose}$ 2·4), which also stained purple and which on acid hydrolysis yielded both amino sugars. This material was further purified by a second chromatographic run with the same solvent system when further small quantities of the free *N*-acetylated amino sugars were removed from it.

(*b*) Bound hexosamine material eluted from Dowex-50 by 0·3N-HCl. Also by chromatography with butanol–acetic acid–water, this material was separated after 4 hr. into three bands which stained reddish purple with Elson–Morgan reagent together with a small amount of material which did not move from the origin. On hydrolysis all three bands which moved were shown to contain both amino acids and amino sugars but only the slowest band contained glucose.

Paper electrophoresis. Materials in the bands on the paper chromatograms were eluted with water and, after concentration of the solutions *in vacuo*, examined by paper electrophoresis.

Oligosaccharide fraction

This fraction (*A* in Fig. 3) was present along with the free *N*-acetylamino sugars and the glucose. The behaviour on the column together with the reaction given in the Reissig *et al.* (1955) *N*-acetylglucosamine test have been taken to indicate that at least part of the amino sugars present is acetylated. Table 2 shows the analytical results obtained

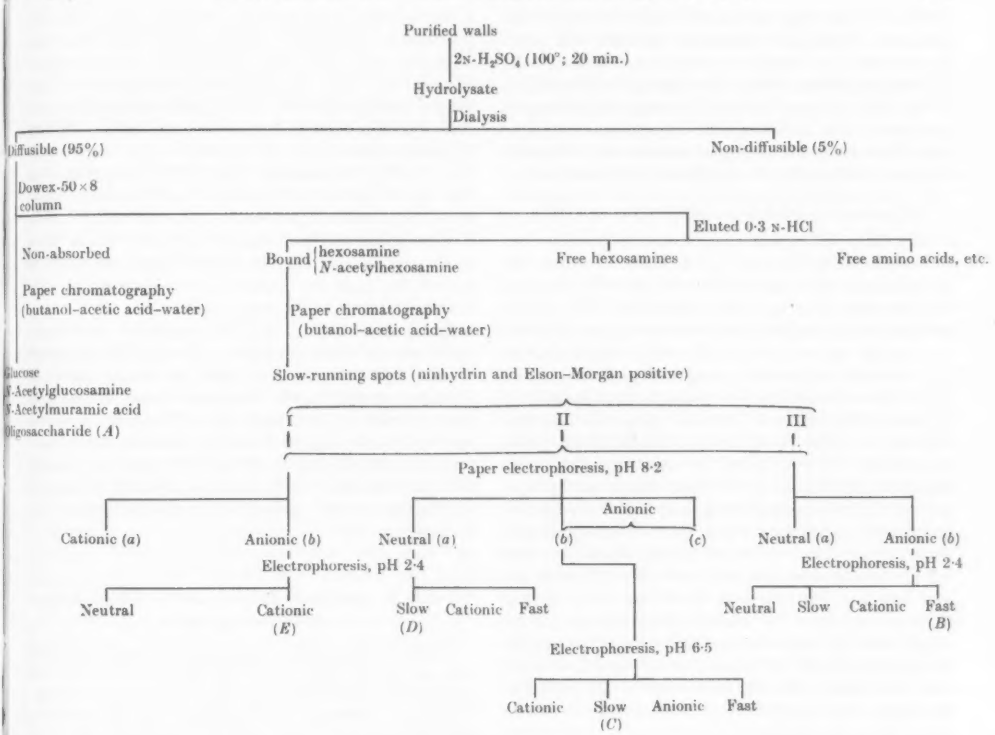


Fig. 3. Fractionation of partial acid hydrolysates of mucopeptide from *M. lysodeikticus*. Compositions of the solvent used for paper chromatography and of the buffers used for paper electrophoresis are given in the Materials and Methods section.

Table 2. *Composition of two oligosaccharide fractions isolated by the methods given in the text (A in Fig. 3)*

Hydrolysis was for 4 hr. with 4N-HCl at 100°. Results are expressed relative to glucosamine. (*a*) and (*b*) represent two separately isolated batches.

	Amount (μmoles)	
	(*a*)	(*b*)
Glucosamine	1·0	1·0
Muramic acid	2·1	2·5
Glycine	0·17	0·20
Alanine	0·08	0·08
Glutamic acid	Trace	0·12
Apparent *N*-acetylhexosamine*	— †	1·50

* *N*-Acetylhexosamine measured on the unhydrolysed material by the Reissig, Strominger & Leloir (1955) test. Result is expressed in terms of *N*-acetylglucosamine.

† Not examined.

after acid hydrolysis of the fraction. The colour given by batch (*b*) in the *N*-acetylhexosamine test was equivalent to about 1·5 μmoles of *N*-acetylglucosamine compared with 1·0 μmole of glucosamine and 2·5 μmoles of muramic acid. The exact significance of this result is difficult to interpret owing to lack of knowledge of the effects of substitution on the intensity of colour given in the *N*-acetylhexosamine test by *N*-acetylmuramic acid. It does, however, suggest that a considerable proportion of one of the acetylhexosamines has a free C-1 position.

In both preparations small amounts of amino acids are also present. These, however, are very small and vary considerably between the two batches. It is not considered that these are likely to be part of the molecule but arise from small amounts of peptides occurring as contaminants.

Some of the oligosaccharide was incubated with lysozyme overnight. When the product was chromatographed in butanol–acetic acid–water and the paper sprayed with Elson–Morgan spray (Partridge, 1948) only a single spot of the original unchanged material could be detected. It appears therefore that the linkage between glucosamine and muramic acid in this material is unaffected by lysozyme.

Muramate–glucosamine–glycine mucopeptide

This material appeared at point *B* in the fractionation scheme shown in Fig. 3. Table 3 shows the composition of hydrolysates obtained by heating the material for 4 hr. with 4N-HCl at 100°. Again two separately isolated batches were examined and the results agreed reasonably well, suggesting an approximate molecular composition of one molecule of glucosamine, one of muramic acid and one of glycine, although one of the values for glycine appears to differ from unity by more than might reasonably be attributed to experimental error. Again small amounts of other amino acids were present. In this instance it is less possible to exclude the amino acids from being part of the molecule. Apart from the values of lysine there is close agreement between the values for the others in the two batches. The amounts, however, are small and if they are part of the same molecule as the amino sugars and glycine a molecular weight within the region of 5000–10 000 would be indicated, which is not consistent with the behaviour of the material on paper and on columns or with its rapid diffusibility through cellophan.

An examination of the free amino end-group of this material by the fluorodinitrobenzene technique failed to reveal any dinitrophenyl glycine, although *N*-dinitrophenyl glucosamine was present. It is therefore concluded that the amino group of the glycine is covalently linked in this compound.

Muramate–alanine–glycine–glutamate–lysine mucopeptide

This material appeared at point *C* (Fig. 3) during the fractionation. Three separate isolations were made and the analyses of acid hydrolysates are recorded in Table 4. The procedures for isolating batches (*b*) and (*c*) were considerably more rigorous than those for batch (*a*) and, as can be seen, glucosamine, which was present in considerable amounts in (*a*), was almost eliminated. Similarly, the proportions of alanine and lysine were considerably increased in batches (*b*) and (*c*) as compared with batch (*a*). Even, however, in the later batches the results do not correspond with any very simple ratio of the component molecules. This may suggest that the compound has a high molecular weight. So far we have not had enough of this material to examine it by physical methods. Alternatively, of course, despite the vigorous purification procedure adopted, the final material obtained may still be a mixture of closely similar compounds. The material was examined for free amino end-groups but only lysine substituted in the ε-amino group could be isolated. Before hydrolysis the compound reacts to give a colour in the *N*-acetylhexosamine test, which suggests that the amino group of muramic acid is acetylated; if so, this would prevent it reacting with fluorodinitrobenzene. The absence of any other free amino group may suggest that the peptide chain is linked through its terminal amino group to the carboxyl group of muramic acid.

Incubation with carboxypeptidase produced little or no effect on this molecule. Even when 0·1 mole of enzyme was used for every mole of glycine present in the compound and incubation was continued overnight at 37° there was no appreciable liberation of amino acid from the mucopeptide. At most only 10% of the alanine was set free and no other amino acid appeared. Likewise incubation with lysozyme left the molecule unchanged.

Table 3. *Analysis of a muramate–glucosamine–glycine mucopeptide*

Isolation and analysis of this compound after hydrolysis with 4N-HCl for 4 hr. at 100° was as described in the text (*B* in Fig. 3). Results are expressed relative to glucosamine. (*a*) and (*b*) represent two separately isolated batches of material.

	Amount (μmoles)	
	(*a*)	(*b*)
Glucosamine	1·0	1·0
Muramic acid	1·2	1·1
Glycine	0·93	0·75
Alanine	0·15	0·16
Glutamic acid	0·11	0·14
Lysine	0·10	0·20

Table 4. *Analysis of a muramate–alanine–glycine–glutamate–lysine mucopeptide*

Isolation and analysis of this compound after hydrolysis with 4N-HCl for 4 hr. at 100° was as described in the text (*C* in Fig. 3). Results are expressed relative to muramic acid, which is taken as 4 μmoles. (*a*), (*b*) and (*c*) represent three separately isolated batches purified by slightly different methods.

	Amount (μmoles)		
	(*a*)	(*b*)	(*c*)
Muramic acid	4·0	4·0	4·0
Alanine	2·9	3·4	3·9
Glycine	2·5	2·2	2·4
Glutamic acid	1·6	1·7	1·7
Lysine	1·1	1·5	1·6
Glycosamine	0·29	Trace	0·19

Other peptide material isolated but not examined in detail

Two other mucopeptide fractions were also isolated (*D* and *E*, Fig. 3) but not studied in great detail. One (*D*) of these contained about four molecules each of muramate and alanine and one molecule of glycine as the major constituents. The other (*E*) contained all the compounds in the original cell-wall mucopeptide except muramate. It is to be noted that this latter fraction had glucose as part of the molecule.

DISCUSSION

Analysis of the wall structure from this organism showed that about 94–97 % of its dry weight can be accounted for in terms of the seven components alanine, glutamate, glycine, lysine, glucose, *N*-acetylglucosamine and *N*-acetylmuramic acid. Thus there is considerable reason for saying that the cell wall of this organism as prepared by the method (Cummins & Harris, 1956) used here, consists only of mucopeptide. If other substances are also involved they must be very small in quantity. The low phosphorus content also indicates that very little teichoic acid can be present, an observation which confirms the work of Baddiley *et al.* (1958), who failed to find polyol phosphates in cell walls from *Micrococcus lysodeikticus*.

One obvious difficulty in studying the undegraded bacterial cell-wall mucopeptides is their complete insolubility in all but disruptive solvents. The result of this intransigence is that the homogeneity of the starting material is unproved and it is impossible to say whether the fragments isolated here have their origin from one or more than one high-molecular-weight compound. The observed variation in the relative amounts of the amino acids and sugars from batch to batch of cell wall points to the need for caution in this respect. However, the present work has shown that all seven component compounds are covalently interlinked and has disposed of the unlikely but possible hypothesis that the original mucopeptide consists entirely of a number of polymers each built from only one or two of the seven components. The composition of the fragments examined in detail shows that in some part of the molecule glucosamine and muramate are joined together. This provides some support for the hypothesis of an amino sugar backbone to the original mucopeptide, as suggested by Brumfitt *et al.* (1958). Lysozyme does not affect this isolated oligosaccharide, which seems to make it unlikely that the major action of lysozyme is to split the bond between the two amino sugars in the absence of further substitution. This is consistent with the statement (Salton, 1956) that a disaccharide of glucosamine and muramate is liberated from the cell-wall mucopeptide by the action of lysozyme.

The second fragment isolated contains the two amino sugars and glycine. No terminal glycine amino group can be demonstrated, which suggests that this group is already linked to some other molecule. The presence of the carboxyl group in muramate makes it tempting to suggest that this is the group with which the amino group of glycine is combined to give a type of peptide. No definite proof for this suggestion exists, however, and it remains possible that the glycine amino group is combined in some way with the hydroxy groups of the sugars: however, no such compounds have been described.

Not only were the oligosaccharide and the glycine–oligosaccharide resistant to the action of lysozyme, but so also was the third, more complicated fragment. Thus so far no fragment less than the whole polymer has been isolated which is susceptible to lysozyme action. The larger fragment was also resistant to the action of carboxypeptidase despite the failure to demonstrate a free amino group. The absence of a reactive amino group may suggest that, in this fragment also, the carboxyl group of muramate is combined with the terminal amino group. The absence of carboxypeptidase action might be explained either by the presence in the molecule of the sugar group or alternatively by the presence of amino acids in the D-configuration, either of which might be expected to prevent hydrolysis by the enzyme.

The total yields of the materials examined have been small. For example, a total of only about 3 % of the weight of the original wall mucopeptide was obtained as at least three different peptides and probably more. Thus it is not yet safe to hypothesize about the structure of the whole wall. It is, however, possible to say that some parts of the muramic acid and glucosamine are linked together and that some of the oligosaccharide is linked to glycine probably by way of the carboxyl group of the muramic acid and the amino group of glycine. Further work depends on finding more satisfactory methods for hydrolysing the wall mucopeptide and better methods for separating the resultant small mucopeptides.

SUMMARY

1. The quantitative composition of mucopeptide from cell walls of *Micrococcus lysodeikticus* is given. About 95 % of the dry weight of the wall preparation can be accounted for.

2. Acid hydrolysis of the mucopeptide has been studied.

3. Three fragments have been isolated from the acid hydrolysates: (*a*) an oligosaccharide containing

glucosamine and muramic acid; (*b*) a mucopeptide containing glycine, muramic acid and glucosamine; (*c*) a mucopeptide containing muramic acid, glycine, lysine, glutamate and alanine.

4. All the fragments isolated are resistant to the action of lysozyme.

5. The significance of the mucopeptide fragments for the structure of the wall mucopeptide is discussed.

We have much pleasure in acknowledging the technical assistance of Ian Mathison and Martyn Hodgson during the course of this work.

REFERENCES

Abrams, A. (1958). *J. biol. Chem.* **230**, 949.
Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Armstrong, J. J., Baddiley, J., Buchanan, J. G. & Carss, B. (1958). *Nature, Lond.*, **181**, 1692.
Baddiley, J., Buchanan, J. G. & Carss, B. (1958). *Biochim. biophys. Acta*, **27**, 220.
Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
Brumfitt, W., Wardlaw, A. C. & Park, J. T. (1958). *Nature, Lond.*, **181**, 1783.
Crumpton, M. J. (1958). *Biochem. J.* **69**, 25P.
Cummins, C. S. & Harris, H. (1956). *J. gen. Microbiol.* **14**, 583.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1955). *Meth. biochem. Anal.* **2**, 360.
Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1952). *Biochem. J.* **51**, 25.
Hundley, J. M., Ing, R. B. & Krauss, R. W. (1956). *Science*, **124**, 535.
Ikawa, M. & Snell, E. E. (1956). *Biochim. biophys. Acta*, **19**, 576.
Jeanes, A., Wise, C. S. & Dimler, R. J. (1951). *Analyt. Chem.* **23**, 415.
Kay, R. E., Harris, D. C. & Entenman, C. (1956). *Arch. Biochim. Biophys.* **63**, 14.
Kent, L. H. (1958). *Biochem. J.* **67**, 5P.
King, E. J. (1947). *Micro Analysis in Medical Biochemistry*. p. 20. London: J. and A. Churchill Ltd.
Kunkel, H. G. (1954). *Meth. biochem. Anal.* **1**, 141.
Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
McCarty, M. (1952*a*). *J. exp. Med.* **96**, 555.
McCarty, M. (1952*b*). *J. exp. Med.* **96**, 569.
Mandelstam, J. & Rogers, H. J. (1958). *Nature, Lond.*, **181**, 956.
Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
Mickle, H. (1948). *J. R. micr. Soc.* **68**, 10.
Newton, G. G. F. & Abraham, E. P. (1954). *Biochem. J.* **58**, 103.
Park, J. T. (1952*a*). *J. biol. Chem.* **194**, 885.
Park, J. T. (1952*b*). *J. biol. Chem.* **194**, 897.
Park, J. T. (1958). *Biochem J.* **70**, 2P.
Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **179**, 585.
Park, J. T. & Strominger, J. L. (1957). *Science*, **125**, 99.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Perkins, H. R. & Rogers, H. J. (1958). *Biochem. J.* **69**, 15P.
Porath, J. (1956). *Biochim. biophys. Acta*, **22**, 151.
Porter, R. R. (1957). *Methods in Enzymology*, vol. 4, p. 221. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955). *J. biol. Chem.* **217**, 959.
Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.
Salton, M. R. J. (1952). *Nature, Lond.*, **170**, 746.
Salton, M. R. J. (1953). *Biochim. biophys. Acta*, **10**, 512.
Salton, M. R. J. (1956). *Biochim. biophys. Acta*, **22**, 495.
Salton, M. R. J. & Horne, R. W. (1951). *Biochim. biophys. Acta*, **7**, 177.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Snell, E. E., Radin, N. S. & Ikawa, M. (1955). *J. biol. Chem.* **217**, 803.
Strange, R. E. & Dark, F. A. (1956). *Nature, Lond.*, **177**, 186.
Strange, R. E. & Kent, L. H. (1959). *Biochem. J.* **71**, 333.
Trevelyan, W. E., Procter, D. D. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Wright, D. H. (1933). *J. Path. Bact.* **37**, 257.
Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.

The Incorporation of Amino Acids into the Cell-Wall Mucopeptide of Staphylococci and the Effect of Antibiotics on the Process

By J. MANDELSTAM and H. J. ROGERS
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 3 *February* 1959)

When washed suspensions of staphylococci in buffered glucose are incubated in the presence of certain single amino acids labelled with ^{14}C, radioactivity is incorporated into the fraction of the cells insoluble in hot trichloroacetic acid (Gale & Folkes, 1953). This process is chloramphenicol-resistant and penicillin-sensitive. Other work (Salton & Horne, 1951; McCarty, 1952*a*, *b*; Salton, 1953; Cummins & Harris, 1956) has shown that, after disruption of Gram-positive bacterial cells, material can be obtained, either by differential centrifuging alone or by a combination of centrifuging and enzyme treatment, which appears, by electron-microscopic examination, to be made up of the cell walls of organisms. Acid hydrolysis and paper chromatography have shown that this material consists preponderantly, in most Gram-positive organisms, of a few amino acids together with

hexoses, glucosamine and another amino sugar, since identified as 3-*O*-α-carboxyethyl-D-glucosamine, called muramic acid (Strange & Dark, 1956; Kent, 1957; Strange & Kent, 1959). In staphylococci it contains lysine, glycine, glutamate and alanine together with glucosamine and muramic acid. This class of substances from cell walls, which will be referred to as mucopeptides, constitutes a high proportion of the hot trichloroacetic acid-insoluble material from bacteria. The present paper demonstrates that the incorporation observed by Gale & Folkes (1953) into staphylococci under condition 1 (i.e. when only a single amino acid was present) was, in fact, almost exclusively due to the growth of the cell wall. A preliminary account of this work has already appeared (Mandelstam & Rogers, 1958) and similar results with another strain of staphylococci have been reported (Hancock & Park, 1958). Gale, Shepherd & Folkes (1958) have since shown that a high proportion of the incorporation of some amino acids, such as glycine, into the cold trichloroacetic acid-insoluble fraction from partially disrupted preparations of staphylococci (Gale & Folkes, 1955) is also into cell-wall material.

The present paper also reports a study of the inhibitory action of penicillin, bacitracin and aureomycin on the process of mucopeptide synthesis.

METHODS

Organisms. Staphylococcus aureus 524 (Rogers, 1953) was grown, with shaking, at 35° in a hydrolysed casein medium supplemented with thiamine (1 μg./ml.) and nicotinic acid (1 μg./ml.) (Rogers, 1945). The bacteria were harvested in the exponential phase of growth when the culture contained about 0·8 mg. dry wt. of bacteria/ml. Bacterial concentrations were determined by opacity measurements in a Spekker absorptiometer, with filter 508, and from a calibration curve of opacity against dry wt./ml.

Conditions of cell-wall synthesis. Bacteria were washed twice with 1% (w/v) glucose in 0·05M-phosphate buffer (prepared from M-KH_2PO_4 brought to pH 7 with NaOH). They were then suspended in the same solution with the addition of amino acids or ammonium salts (see below) and incubated in 1 l. conical flasks, with shaking, at 35°. The bacterial density was 1·0–1·03 mg. dry wt./ml. and the volume 130 ml. After the incubation period (usually 1 hr.) the contents of the flask were centrifuged, the bacteria were washed once with 40 ml. of 1% (w/v) glucose in buffer and mucopeptide was isolated by the following method, which is essentially that of Cummins & Harris (1956).

Preparation of mucopeptide. Chilled bacterial suspension containing about 150 mg. dry wt. of bacteria was added to Ballotini beads (10 ml.; grade 12, Griffin and George Ltd., London) and shaken in a Mickle disintegrator for 15 min. at 2°. The suspension was filtered through a grade 1 sintered-glass filter to remove the beads and the filter washed twice with water (10 ml.).

The combined filtrate was centrifuged at 10 000 rev./min. for 10 min. in 50 ml. Lusteroid tubes on an MSE centrifuge model Angle-13 (Measuring and Scientific Equipment Ltd.). The sediment, consisting of cell wall and unbroken cells, was immediately suspended in water (5 ml.) and heated at 100° for 20 min. Omission of this step led to loss of much of the mucopeptide through the action of lytic enzymes (see Mitchell & Moyle, 1957; Richmond, 1959). The preparation was then incubated for 3 hr. at 35° with 1 mg. of trypsin and 1 mg. of ribonuclease in 5 ml. of 0·05M-phosphate buffer, pH 7·6. The remaining wall material was washed once with 10 ml. of water and resuspended in 5 ml. of 0·5N-HCl; 1 mg. of crystalline pepsin was added and the preparation was incubated at 35° overnight. The washed mucopeptide was suspended in about 0·5 ml. of water and acetone (5 ml.) was added. The flocculent material was sedimented by centrifuging at low speed, washed with acetone (5 ml.) and ether (5 ml.), dried at room temperature and finally heated at 100° for 1 hr. to remove traces of water. The product was a white powder. The yield of mucopeptide from freshly harvested cells was 10–12·5 mg./100 mg. dry wt. of bacteria.

In those experiments where mucopeptide had been synthesized in the presence of a ^{14}C-labelled amino acid, all washing and digestion procedures were carried out in the presence of the corresponding unlabelled amino acid at a concentration of 2·5 mg./ml.

Isolation of protein. The supernatant obtained after disintegration of the bacteria and removal of cell wall and whole cells was treated with an equal volume of 10% (w/v) trichloroacetic acid. The precipitate was extracted with hot 5% (w/v) trichloroacetic acid and then with acetone, ethanol–ether and ether (see Mandelstam, 1958*a*). The protein accounted for 30–35% of the dry wt. of the bacteria. In some experiments the whole cells were treated in this manner so that the final product contained both mucopeptide and protein. Such preparations will be referred to as 'mixed trichloroacetic acid precipitates', and accounted for about 50% of the dry wt. of the bacteria.

Composition of mucopeptide. For amino acid determination, 3–4 mg. of mucopeptide was heated at 100° in a sealed tube with 1 ml. of 4N-HCl for 18 hr. The hydrolysate was dried in a vacuum desiccator containing conc. H_2SO_4 and solid NaOH. To remove traces of HCl the residue was twice dissolved and re-evaporated, about 0·4 ml. of water being used each time. The hydrolysate was dissolved in 0·48 ml. of water and 0·08 ml. amounts were applied (from a graduated 0·1 ml. pipette) to Whatman no. 3 paper as a line about 4 cm. long. Amino acid standards at two concentrations (50 and 100 μg.) were included on each sheet, and the chromatograms developed with butanol–pyridine–water (6:4:3, by vol.; Jeanes, Wise & Dimler, 1951) for 48 hr. At the end of this time the four amino acids of the mucopeptide were well separated from one another and from the hexosamines. Amino acids were measured by the following modification of the method of Kay, Harris & Entenman (1956). The sheets were thoroughly dried, sprayed with 0·6% ninhydrin in butanol and heated at 50° for 1 hr. The spots were cut out and transferred to test tubes. The colour was eluted with 70% (v/v) ethanol and read in the Spekker absorptiometer with an Ilford Filter 604 (spectrum green) and 1 cm. micro cells. Estimations were carried out at least in duplicate and were reproducible to within ±5%.

Glucosamine and muramic acid were determined as described by Perkins & Rogers (1959).

Free amino acid pool. Washed bacteria (10–20 mg. dry wt.) were suspended in 10 ml. of water and heated at 100° for 20 min. to extract free amino acids. Subsequent procedures for separating and estimating the components of the pool were as described by Mandelstam (1958*b*).

Measurement of radioactivity. Radioactivity of protein, mucopeptide and 'mixed trichloroacetic acid precipitates' was measured on 0·3 cm.² polythene disks at infinite thickness (20 mg./cm.²) with a thin end-window Geiger–Müller counter. Counting was continued until the count was 1000 above the background.

Specific activity of glycine from hydrolysates of protein was determined after isolation of the glycine on paper with water-saturated M-cresol (Mandelstam, 1958*a*). Estimation of specific activity of glycine in mucopeptide was carried out in the same way except that the paper chromatograms were developed with the butanol–pyridine–water solvent described above.

RESULTS

Biosynthesis of mucopeptide from glucose and glycine in the presence of chloramphenicol

Washed staphylococci were incubated, with shaking, in buffer–glucose solution (see Methods section) containing (per ml.) 200 μg. of [1-^{14}C]glycine (specific activity 20 counts/min./μg.) and 50 μg. of chloramphenicol. A control flask without chloramphenicol was incubated at the same time. The opacity of both suspensions increased by 30 % during the experiment.

At the end of 1 hr. 20 ml. samples were taken from each flask and centrifuged. The bacteria were washed and treated with trichloroacetic acid to give 'mixed trichloroacetic acid precipitate'. Mucopeptide and cytoplasmic protein were prepared from the remainder of the suspension and the radioactivity present in each fraction was determined.

It was found (Table 1) that, with the control cells, the specific activity of the mucopeptide was some 20 times as great as that of the protein. Chloramphenicol had little or no effect upon incorporation of radioactivity into mucopeptide but depressed the small incorporation into protein. The specific activity of the 'mixed trichloroacetic acid precipitates' is what would be expected on the assumption that the mucopeptide constitutes about one-third of the weight of the material in this fraction.

Radioactivity measurements made in this way could be misleading, if, during the incubation, some of the labelled glycine had been converted into other amino acids and these had then been incorporated. For this reason, samples of the fractions were hydrolysed, the glycine was isolated and its specific activity determined.

The results (Table 2) confirm those obtained by measuring the radioactivity of the unhydrolysed materials. The glycine in the protein fraction is 2 % labelled, i.e. its specific activity is 2 % of that of the glycine in the incubation medium. By contrast, the glycine of the mucopeptide is 41 % labelled. Glutamic acid, alanine, lysine and hexosamine separated from the same hydrolysate all had negligible radioactivity and together accounted for about 5 % of the total radioactivity of the mucopeptide. The degree of labelling of the glycine is that which would be expected if the mucopeptide had increased by about 70 % during the incubation. It will be shown (see below) that a net increase of this order does, in fact, occur under these conditions.

It is apparent that chloramphenicol has little effect upon the synthesis of mucopeptide.

Table 1. *Distribution of radioactivity in various cell fractions*

Washed staphylococci were incubated for 1 hr. with glucose (1 %, w/v) and [^{14}C]glycine (200 μg./ml.; specific activity 20 counts/min./μg.) in the presence and in the absence of chloramphenicol (70 μg./ml.). Protein and mucopeptide were isolated after mechanical disintegration of the bacteria (see Methods section).

	Specific activity (counts/min./mg.)	
	Control	With chloramphenicol
'Mixed trichloroacetic acid ppt.' (protein + mucopeptide)	767	727
Protein	126	62
Mucopeptide	2518	2472

Table 2. *Specific activity of glycine isolated from acid hydrolysates of cell fractions from staphylococci*

Cells were incubated with glucose and [^{14}C]glycine (see protocol of Table 1), with and without chloramphenicol. In the last column, the specific activity of the glycine from the hydrolysate is expressed as a percentage of that of the glycine added to the incubation medium.

	Glycine (μg.)	Counts/min./μg.	Counts/min./μg. of glycine	Percentage labelling
Without chloramphenicol				
'Mixed trichloroacetic acid ppt.'	242	594	2·5	12·5
Protein	160	67	0·4	2
Mucopeptide	225	1839	8·2	41
With chloramphenicol				
'Mixed trichloroacetic acid ppt.'	176	418	2·4	12
Protein	220	63	0·3	1·5
Mucopeptide	220	1645	7·5	37·5

Requirements for mucopeptide synthesis

The requirements for mucopeptide synthesis were investigated by incubating washed staphylococci for 1 hr. in buffer solution with glucose and one or more of the four amino acids which it normally contains. Owing to the difficulty of handling more than about four samples at a time the results shown in Table 3 have been drawn from several experiments.

Freshly harvested cells contained 9–12 mg. of mucopeptide/100 mg. dry wt. of bacteria and this was increased by a small and variable amount if the cells were incubated with buffer and glucose alone (see Table 3). Addition of DL-alanine, DL-glutamic acid or DL-lysine singly produced a significant increase (up to 25 %) in the amount of mucopeptide. A more marked effect was found with glycine, which uniformly caused a net synthesis of over 50 % in 1 hr. It will be seen that the further addition of glutamic acid or lysine in addition to glycine did not greatly increase the yield of mucopeptides. However, the inclusion of all four amino acids in the incubation medium generally produced an increase of 100–150 %.

Relationship between opacity and synthesis of mucopeptide in washed suspensions

In the type of experiments which we have described, it was found that there was always some increase in the opacity of those bacterial suspensions in which there was a net increase in the mucopeptide content of the cells. Amino acid incorporation (Tables 1 and 2) indicated negligible protein synthesis under these conditions, and analysis of nucleic acid content of the cells, by measuring the absorption at 260 mμ of material extracted by hot trichloroacetic acid (Schneider, 1945), showed that there was an increase of the order of 30 % in nucleic acid content. In spite of this increase in nucleic acid, mucopeptide synthesis was mainly responsible for the increased opacity of the suspensions. The results of a number of experiments were analysed by plotting the actual increase in mucopeptide against the apparent increase in mass, obtained from opacity measurement and the standard calibration curve for the organism. The points lie about a smooth curve, which can be used as a rough indication of mucopeptide synthesis (Fig. 1). The only experiments in which any serious discrepancy was observed between the weight of mucopeptide formed and that expected from opacity measurement were those in which a high concentration (1000 units/ml.) of penicillin was used. In these experiments the increase in mucopeptide was about 50 % less than that expected from the change in opacity (see below).

Table 3. *Conditions for the synthesis of mucopeptide*

Washed staphylococci were incubated for 1 hr. in buffer solution with glucose (1 %, w/v) and one or more amino acids at a final concentration of 400 μg./ml. The bacteria were disintegrated and the mucopeptide was isolated and weighed. Freshly harvested cells contained 9–12 mg. of mucopeptide/100 mg. dry wt. of bacteria.

Additions	Increase in mucopeptide (%)
None	0–10
DL-Lysine	20
DL-Glutamic acid	25
DL-Alanine	20
Glycine	55–80
Glycine + DL-glutamic acid	60
Glycine + DL-lysine	80
Glycine + DL-lysine + DL-glutamic acid + DL-alanine	100–150

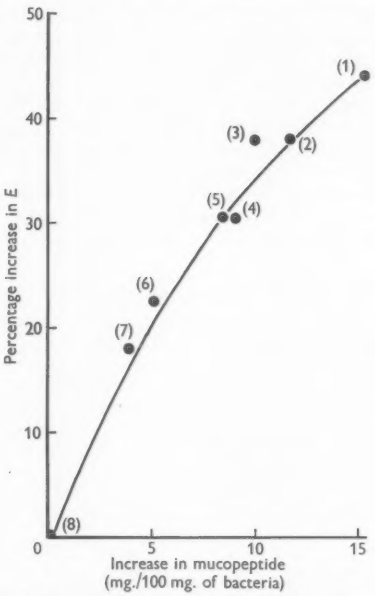


Fig. 1. Relationship between extinction and mucopeptide synthesis. Washed staphylococci were incubated for 1 hr. in buffer solution containing 1 % (w/v) of glucose and additions as listed below. Samples were taken at the beginning and end of the incubation for isolation of mucopeptide (see Methods section). The increase in mucopeptide has been plotted against the apparent increase in bacterial mass obtained by measurement of extinction. Additions to the glucose–buffer solution: (1) Four amino acids (i.e. glycine, DL-alanine, DL-glutamic acid and DL-lysine, each at 400 μg./ml.). (2) and (3) As (1). (4) NH_4Cl (1 mg./ml.). (5) Glycine (400 μg./ml.). (6) As (1) + penicillin (100 units/ml.). (7) As (1) + bacitracin (100 μg./ml.). (8) None.

Free amino acid content of staphylococci in relation to the synthesis of mucopeptide

The foregoing experiments suggested that glycine might be the limiting factor in mucopeptide synthesis by washed staphylococci incubated with glucose alone. Preliminary experiments showed that the amount of free glycine in the 'pool' from freshly harvested cells was less than that of the other amino acid components of mucopeptide, and also indicated that the glycine might be acting as a source of nitrogen for the synthesis of the other three amino acids.

This conclusion was tested by the following experiment. Washed staphylococci were incubated for 1 hr. in the usual buffer–glucose solution (130 ml.) containing (*a*) no addition, (*b*) NH_4Cl (1 mg./ml.) and (*c*) glycine (400 μg./ml.). A fourth flask, similar to (*a*), was set up for the zero-time sample. Duplicate 15 ml. samples were taken from each flask for analysis of the free amino acids. The rest of the bacterial suspension was used for the preparation of mucopeptide.

It was found that, whereas there was no synthesis in the presence of glucose alone, incubation with NH_4Cl or glycine led to a net increase of about 60 % in the mucopeptide content (Table 4).

The analysis of the amino acid pool is given in Table 5. Incubation of the cells with glucose produced a diminution in the concentration of all four amino acids. This was most noticeable for glutamic acid, which dropped from 3·32 to 0·32 mg./100 mg. of bacteria. Even if this dissipation of pool material had not taken place, it is obvious that, with the exception of glutamic acid, there was not enough of each amino acid at zero time to account for the quantities incorporated into the mucopeptide during the experiment. A comparison of the zero-time column and the final column of Table 5 shows that this deficit is most manifest for glycine and hexosamine, which together constitute 60 % of the mucopeptide.

When NH_4Cl was present, not only was there synthesis of mucopeptide (7·6 mg./100 mg. of bacteria) but the concentrations of the free amino acids (lysine excepted) and hexosamine increased. The results with glycine were broadly similar to those obtained with NH_4Cl. The main differences were that the concentrations of glutamic acid and hexosamine were lower, and that of glycine was, as might be expected, a great deal higher.

Composition of mucopeptide

Analysis of mucopeptide isolated from cells harvested at about 0·8 mg. dry wt. of bacteria/ml. of culture was carried out by the techniques described in the Methods section.

The main amino acid components were lysine, glycine, glutamic acid and alanine (no attempt has been made to determine the optical configuration of these amino acids). In addition glucosamine and muramic acid were found and, if it is assumed that they were in the *N*-acetylated form, over 90 % of the mucopeptide preparation could be accounted for. The remainder consisted of small and variable amounts of other amino acids. Valine, serine and leucine (or isoleucine) were each present to the extent of 0·5–2 %. In addition, similar amounts of aspartic acid were found in some of the preparations, as well as occasional traces of phenylalanine.

Table 4. *Mucopeptide content of staphylococci*

Incubation was for 1 hr. in buffer–glucose solution with additions: (*a*) none; (*b*) NH_4Cl (1 mg./ml.); (*c*) glycine (400 μg./ml.).

	Mucopeptide (mg./100 mg. of bacteria)
Zero-time sample	12·2
(*a*) No addition	12·0
(*b*) NH_4Cl	19·8
(*c*) Glycine	20·2

Table 5. *Free amino acids during synthesis of mucopeptide*

Washed staphylococci were incubated for 1 hr. with buffer–glucose solution with additions: (*a*) none; (*b*) NH_4Cl (1 mg./ml.); (*c*) glycine (400 μg./ml.). Samples for free amino acid analysis were taken before and after incubation. It has been assumed that the hexosamine was present as glucosamine. The last column shows the amount of each amino acid required for the mucopeptide synthesized in flask (*c*) during the experiment (7·6 mg./100 mg. of bacteria). The corresponding quantities for (*b*) are 5% lower. The values have been calculated assuming the following composition of mucopeptide: lysine, 7%; glutamic acid, 12%; glycine, 20%; alanine, 14%; hexosamine, 43%.

	Amino acid (μg./100 mg. dry wt. of bacteria)				
	Zero-time	(*a*)	(*b*)	(*c*)	Required for mucopeptide
Lysine	170	120	130	130	560
Glutamic acid	3320	320	5730	1620	960
Glycine	40	20	80	1920	1600
Alanine	970	750	1420	1270	1120
Hexosamine	—	—	200	70	3400
Total	4500	1210	7560	5010	7640

Table 6. *Composition of mucopeptide isolated from five different cultures of staphylococci*

Cells were harvested during exponential growth. Values are given as percentages, and those for glucosamine and muramic acid refer to the *N*-acetylated amino sugars.

	1	2	3	4	5	Mean
Glutamic acid	11·6	14·5	11·7	12·7	10·5	12·2
Glycine	17·5	23·1	21·1	20·5	18·6	20·1
Alanine	12·5	16·0	14·2	13·8	12·6	13·8
Lysine	6·2	8·5	8·0	6·9	6·3	7·2
Glucosamine	15·0	—	—	16·9	18·7	16·9
Muramic acid	31·0	—	—	24·0	24·4	26·6
Total (four amino acids)	47·8	62·1	55·0	53·9	48·0	53·3
Total (two amino sugars)	46·0	—	—	40·9	43·1	43·5

The detailed quantitative analysis of five such preparations is given in Table 6. Variations exceeding that of the analytical error were encountered from one batch of mucopeptide to another, and the values in the table have been chosen to cover the extremes of the range. The four main amino acids together constituted 44–62 % of the preparation, and the two amino sugars 40–47 %. In spite of the variation, the total accounted for by these six substances was invariably 90–95 % of the particular sample. The final column of the table gives the mean values, and from these the molar ratios have been calculated, glutamic acid being taken as unity. The values are:

glutamic acid : glycine : alanine : lysine : glucosamine : muramic acid
1 : 3·3 : 1·9 : 0·6 : 1·0 : 1·1

Analyses were also carried out on mucopeptide synthesized in the presence of (*a*) NH_4Cl, (*b*) glycine, (*c*) glycine and the other three amino acids (added in DL form). The values fall within the range shown in Table 6.

Effect of penicillin, bacitracin and aureomycin upon synthesis of mucopeptide

The experiments already described showed that staphylococci in the presence of glucose and the four amino acids provide a convenient system for studying mucopeptide synthesis in isolation from protein synthesis. The effect of penicillin upon mucopeptide synthesis was investigated by incubating washed cells in buffer–glucose solution containing glycine, DL-alanine, DL-glutamic acid and DL-lysine (each at a final concentration of 400 μg./ml.) and benzyl penicillin (100 and 1000 units/ml.). These high concentrations of penicillin were necessary because, in this strain of staphylococci, penicillinase formation can be induced by penicillin. In addition, chloramphenicol (70 μg./ml.) was added to prevent formation of the enzyme. The control flask contained the amino acids and chloramphenicol.

The flasks were shaken for 1 hr. and the mucopeptide was isolated. Also, during the incubation, the extinction of the suspensions was measured at intervals.

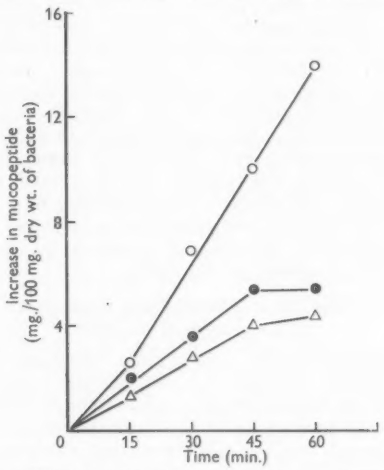


Fig. 2. Effect of penicillin upon synthesis of mucopeptide. Washed staphylococci were incubated in buffer solution containing 1 % (w/v) of glucose and glycine, DL-alanine, DL-glutamic acid and DL-lysine (each at 400 μg./ml.). Chloramphenicol (70 μg./ml.) was added to prevent induced formation of penicillinase. Increase in mucopeptide was obtained from measurements of extinction and the calibration curve shown in Fig. 1. ○, Control; ●, penicillin (100 units/ml.); △, penicillin (1000 units/ml.).

The synthesis of the mucopeptide in the control flask appeared to be linear after the first 15 min. (Fig. 2). In the presence of 100 units of penicillin/ml. a lowered rate was detectable from the start, and synthesis had more or less ended after 45 min. This concentration of penicillin appeared to be sufficient to obtain a maximal effect, since a tenfold increase in the amount of penicillin did not greatly increase the degree of inhibition. In subsequent experiments (see below) penicillin was used at 500 units/ml.

In Table 7 the increase in mucopeptide indicated by the extinction at the end of 1 hr. has been compared with the actual increase determined by weighing. The two methods of estimation agree very well for the control culture and that containing 100 units of penicillin/ml., but the opacity increase obtained with 1000 units/ml. indicates the synthesis of about twice as much mucopeptide as was actually recovered at the end of the experiment.

The effect of bacitracin (100 μg./ml.) was examined in the same way, by incubating cells with the amino acids in glucose–buffer solution; chloramphenicol was omitted. For the first 15 min., mucopeptide synthesis, as measured by extinction, was the same in the presence and in the absence of antibiotic. After this, the rate declined progressively so that at the end of an hour the amount of mucopeptide synthesized was about one-half of that obtained in the control culture (Fig. 3). In a similar experiment, in which the mucopeptide was isolated and weighed, it was found that synthesis was inhibited 58 % by bacitracin.

The effect of aureomycin (100 μg./ml.) was also investigated. At this concentration no mucopeptide synthesis was detectable.

Composition of mucopeptide synthesized in the presence of penicillin

Analysis of the mucopeptide estimated after incubation of cells with four amino acids and penicillin (see above) gave a low value for alanine (11·0 %, compared with 12·7 % for the control) and suggested that the antibiotic might interfere not only with the amount of mucopeptide synthesized but also with its composition. Any such effect is obviously obscured by the fact that the major part (about 70 %) of the sample analysed consists of normal material which was present at the beginning of the experiment. The newly synthesized mucopeptide might be expected to show a divergence from the normal value about two to three times as great as that apparent from the crude analytical figures.

In an attempt to obtain some definite evidence on this point, experiments of the following type were done with labelled amino acids. Washed staphylococci were incubated as before in flasks containing buffer–glucose with chloramphenicol and 400 μg./ml. of each of the four amino acids previously used. In the first flask the alanine was labelled (14 500 counts/min./μmole). Another flask had the same additions, except that the glutamic acid was labelled instead of the alanine (15 140 counts/min./μmole). After incubation for 1 hr. mucopeptide was isolated from both suspensions and the radioactivity measured. The activity (counts/min./mg.) in the alanine- and glutamate-labelled mucopeptides was 307 and 179, giving a ratio of 1·72. Correction of this value for the difference in molar specific activity of the two amino acids gives a ratio of alanine:glutamic acid 1·8:1. Within experimental error this is the ratio found by chemical analysis for normal mucopeptide. Mucopeptide, however, isolated from a similar pair of cultures containing penicillin (500 units/ml.) gave a ratio of 1·4:1. In another experiment the ratio was even lower (0·9:1). The

Table 7. *Effect of penicillin on synthesis of mucopeptide*

Washed staphylococci were incubated in buffer–glucose with glycine, DL-alanine, DL-glutamic acid and DL-lysine (each at 400 μg./ml.) in the presence and in the absence of penicillin. Chloramphenicol (70 μg./ml.) was present to prevent induced formation of penicillinase. The amount of mucopeptide synthesized after incubation for 1 hr. has been estimated from increase in extinction of the bacterial suspension and also by weighing.

	Increase in mucopeptide (mg./100 mg. of bacteria)	
	Extinction	Weight
Control	9·0	8·5
Penicillin (100 units/ml.)	5·5	4·8
Penicillin (1000 units/ml.)	4·3	2·0

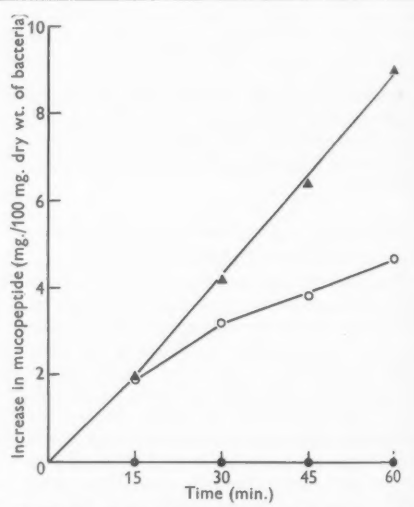


Fig. 3. Effect of bacitracin and aureomycin upon synthesis of mucopeptide. Washed staphylococci were incubated in 1 % (w/v) glucose–buffer solution containing glycine, DL-alanine, DL-glutamic acid and DL-lysine (each at 400 μg./ml.). Increase in mucopeptide was obtained from measurement of extinction and the calibration curve shown in Fig. 1. ▲, Control; ○, bacitracin (100 μg./ml.); ●, aureomycin (100 μg./ml.).

analyses with labelled amino acids therefore confirm those carried out by chemical methods and show that there is a fall in the ratio of alanine to glutamic acid in mucopeptide formed in the presence of penicillin.

Preliminary results suggested that there might also be a decrease in the ratio of glycine to glutamic acid, but these observations have not been pursued.

DISCUSSION

If it is assumed that the amino acids extractable from the cells by hot water can be used directly for cell-wall synthesis, and either have the right optical configuration or can be converted rapidly by the action of the appropriate racemases, there is sufficient glutamic acid and alanine present to allow the synthesis of 7–8 mg. of mucopeptide/100 mg. of cells. The amount of extractable lysine is enough for about one-third of this amount of mucopeptide, but there is little or no available glycine or hexosamine. This deficiency of glycine in the 'pool' would alone be sufficient to explain, qualitatively, the greater incorporation of glycine and the more active cell-wall synthesis which occurs when only this amino acid is added. The quantitative experiments reported here show that, with our strain of staphylococci, glycine can also serve as a source of nitrogen for the synthesis of other amino acids, as, in fact, can the NH_4^+ ion. Whether or not the carbon of glycine can also be used for amino acid synthesis cannot be determined from these experiments, owing to the presence of large amounts of unlabelled carbon fragments arising from the glucose. That the breakdown of formed protein does not give sufficient amino acids to affect the results is due to the slow rate at which this process occurs, i.e. 1–2 %/hr. (J. Mandelstam, unpublished observations).

Although the formation of both proteins and mucopeptide must involve peptide-bond synthesis, these two processes can be readily dissociated. If the cells are incubated either with any one of the amino acids present in the cell wall together with glucose, or with a full complement of amino acids, glucose and chloramphenicol, no significant protein synthesis occurs, whereas cell-wall mucopeptide is formed. Contrariwise, if penicillin is added, mucopeptide formation is inhibited; but protein synthesis can continue (Gale & Folkes, 1953; Strominger, 1957). Park (1958) has reported similar results. Aureomycin stops the formation of proteins, mucopeptide and nucleic acid.

It is to be noted that penicillin does not stop the biosynthesis of mucopeptide immediately; synthesis continues for about 45 min. at a reduced rate before it ceases. This long period of delay would seem unlikely to be due either to slow penetration of the antibiotic to the synthesizing sites or to its destruction by small amounts of penicillinase in the cells. For the former possibility the period is too long and if the latter were true one might have expected an initial period of inhibition followed by an acceleration in synthesis. It is also difficult to understand the period of delay in terms of the exhaustion of precursors of the process, if mucopeptide 'biosynthesis' is to be explained in terms of a single invariable pathway. If, however, two independent pathways leading either to more than one compound or to one compound of variable composition are presupposed, then the kinetics of action of the antibiotic are comprehensible. One step in one pathway may be inhibited immediately, thus stopping synthesis by this route, whilst synthesis can still proceed by the second route, either until the supply of its intermediates is exhausted or until metabolic imbalance of some sort becomes too great for the cell. Such a hypothesis is supported by our finding that the mucopeptide synthesized in the presence of penicillin has a different composition from that in the control cells. Owing to the difficulty of finding solvents for the mucopeptide which are not likely to hydrolyse covalent bonds, homogeneity tests have so far been impossible.

Strominger & Park (1957) have suggested that the mechanism of penicillin action is to prevent further incorporation of the peptide, isolated by Park (1952*a*, *b*, *c*), into cell wall. If this hypothesis were wholly correct then one might expect synthesis of mucopeptide to stop almost immediately on the addition of penicillin. This does not occur, and a search for peptides of the type isolated by Park in the cells of our strain of staphylococci, inhibited by penicillin, has been unsuccessful (unpublished observations), so that, if present, their concentration must be very low.

An alternative to the present suggestion of a dual mechanism for normal mucopeptide synthesis might be that the presence of penicillin causes a different mucopeptide to be synthesized by way of a partially resistant pathway. This process may be slower than the normal and may cease when its intermediates are exhausted.

One result of this study has been to make unnecessary, at least in this system, the intellectually difficult hypothesis of exchange of amino acids in formed protein. Because radioactive amino acids were incorporated into staphylococcal 'mixed protein' under conditions where no net increase in protein could be demonstrated, Gale & Folkes (1953) and Gale (1957) proposed that the amino acids in the already formed protein exchanged with the exogenous supply. It has now been shown that the incorporation into whole cells which occurred under these authors' conditions was entirely due to

synthesis of cell-wall mucopeptide. Whether or not other substances, such as teichoic acid (Baddiley, Buchanan & Carss, 1958; Armstrong, Baddiley, Buchanan & Carss, 1958), which are associated with isolated cell walls in some organisms, are also synthesized under these conditions has not been tested. The method of mucopeptide preparation selected here is likely to remove them from the cell wall, and also the values for the ratioactive content of the 'mixed trichloroacetic acid precipitates' agree with those for the isolated mucopeptide. Again, the treatment with hot 5% trichloroacetic acid in the preparation of mixed precipitates would certainly be expected to remove any teichoic acid.

SUMMARY

1. Staphylococci incubated in the presence of ^{14}C-labelled glycine, glutamate, alanine or lysine, either singly or together, incorporate these almost exclusively into the cell-wall mucopeptide.

2. This incorporation can be entirely accounted for by the increase in mass of cell-wall mucopeptide. This process is resistant to chloramphenicol.

3. The amino acids can be replaced by glucose and ammonium chloride with the strain of *Staphylococcus aureus* used.

4. The synthesis of cell-wall mucopeptide is inhibited by penicillin and bacitracin, neither of which inhibits protein synthesis.

5. Some evidence is presented which suggests that the small amount of mucopeptide synthesized in the presence of penicillin has an abnormal composition.

6. The relation of these observations to the mechanism of penicillin action and to a hypothesis concerned with protein synthesis is discussed.

We are indebted to Ruth Coyle and Ian Mathison for technical assistance given during the course of this work.

REFERENCES

Armstrong, J. J., Baddiley, J., Buchanan, J. G. & Carss, B. (1958). *Nature, Lond.*, **181**, 1692.
Baddiley, J., Buchanan, J. G. & Carss, B. (1958). *Biochim. biophys. Acta*, **27**, 220.
Cummins, C. S. & Harris, H. (1956). *J. gen. Microbiol.* **14**, 583.
Gale, E. F. (1957). *Symp. biochem. Soc.* **14**, 47.
Gale, E. F. & Folkes, J. P. (1953). *Biochem. J.* **53**, 493.
Gale, E. F. & Folkes, J. P. (1955). *Biochem. J.* **59**, 661.
Gale, E. F., Shepherd, C. J. & Folkes, J. P. (1958). *Nature, Lond.*, **182**, 592.
Hancock, R. & Park, J. T. (1958). *Nature, Lond.*, **181**, 1050.
Jeanes, A., Wise, C. S. & Dimler, R. J. (1951). *Analyt. Chem.* **23**, 415.
Kay, R. E., Harris, D. C. & Entenman, C. (1956). *Arch. Biochem. Biophys.* **63**, 14.
Kent, L. H. (1957). *Biochem. J.* **67**, 5P.
McCarty, M. (1952*a*). *J. exp. Med.* **96**, 555.
McCarty, M. (1952*b*). *J. exp. Med.* **96**, 569.
Mandelstam, J. (1958*a*). *Biochem. J.* **69**, 110.
Mandelstam, J. (1958*b*). *Biochem. J.* **69**, 103.
Mandelstam, J. & Rogers, H. J. (1958). *Nature, Lond.*, **181**, 956.
Mitchell, P. & Moyle, J. (1957). *J. gen. Microbiol.* **16**, 184.
Park, J. T. (1952*a*). *J. biol. Chem.* **194**, 877.
Park, J. T. (1952*b*). *J. biol. Chem.* **194**, 885.
Park, J. T. (1952*c*). *J. biol. Chem.* **194**, 897.
Park, J. T. (1958). *Biochem. J.* **70**, 2P.
Perkins, H. R. & Rogers, H. J. (1959). *Biochem. J.* **72**, 647.
Richmond, M. (1959). *Biochim. biophys. Acta*, **31**, 564.
Rogers, H. J. (1945). *Biochem. J.* **39**, 435.
Rogers, H. J. (1953). *J. Path. Bact.* **66**, 545.
Salton, M. R. J. (1953). *Biochim. biophys. Acta*, **10**, 512.
Salton, M. R. J. & Horne, R. W. (1951). *Biochim. biophys. Acta*, **7**, 177.
Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
Strange, R. E. & Dark, F. A. (1956). *Nature, Lond.*, **177**, 186.
Strange, R. E. & Kent, L. H. (1959). *Biochem. J.* **71**, 333.
Strominger, J. L. (1957). *J. biol. Chem.* **224**, 509.
Strominger, J. L. & Park, J. T. (1957). *Science*, **125**, 99.

Observations on the Composition and Metabolism of the Nitrogen-Fixing Root Nodules of *Myrica*

By G. LEAF, ISOBEL C. GARDNER and G. BOND
Departments of Biochemistry and Botany, The University of Glasgow

(*Received* 4 *December* 1958)

In an earlier study of the fate of labelled nitrogen fixed by the root nodules of *Alnus* (Leaf, Gardner & Bond, 1958) the greatest abundances of ^{15}N were found in the carbamyl group of citrulline and the amino group of glutamic acid. This was taken to indicate that an early product of fixation was possibly ammonia, and that though the pattern of nitrogen metabolism in *Alnus* differs in other respects from that of the leguminous plants and other nitrogen-fixing organisms, their systems for nitrogen fixation had this much in common. This paper describes similar work undertaken with the root nodules of *Myrica*, which have also been shown to fix nitrogen (Bond, 1951, 1958).

METHODS

Source of nodule material. Nodulated plants of *Myrica gale* L. (bog myrtle) were raised in the greenhouse in water culture in Crone's solution (Bond, 1951) (pH 5–6), free of combined nitrogen except for a very small amount provided during the period of nodule development. The latter was induced by inoculating the roots of the seedlings with a suspension of crushed *Myrica* nodules collected from the field. In the early stages of plant growth the Crone's solution was supplied at one-quarter of the normal concentration, and was later increased to one-half of the latter; growth was much superior to that obtained on previous occasions when the full strength solution was employed.

Exposure to excess of ^{15}N. Exposure of nodules to excess of ^{15}N was effected in the summer of the second year of growth of the plants, at a stage when the latter were some 40 cm. in height. The nodules were rapidly detached from the plants, dried roughly with filter paper, and samples of approximately 1·3 g. placed, together with 1 ml. of culture solution, in Universal Containers of capacity 25 ml. These were immediately charged to atmospheric pressure with a gas mixture containing 10% of nitrogen (with 36 atoms % ^{15}N), 20% of oxygen and 70% of argon. Exposures were made on three different days in the period 1–9 August, a total of about 10 g. of nodules being taken on each occasion. The exposures were begun at the same hour (3 p.m.) on each day, and on different days varied in duration from 2 hr. to 45 min.; during exposure the nodules were held at a temperature of approximately 25°, and were subsequently bulked and plunged into solid carbon dioxide.

Extraction. The nodules were extracted with 9 vol. of 0·1 N-HCl in a blender cooled in ice. The suspension was centrifuged at 1000 *g* for 20 min. at 0° and the extract lyophilized.

Fractionation. An extract of *Myrica* nodules was examined by two-dimensional paper chromatography, with butanol–acetic acid–water (4:1:5, by vol.) followed by saturated aqueous phenol in the presence of ammonia, and a preliminary quantitative determination of its composition was made by the method of Moore & Stein (1954), which was used for alder extracts (Leaf *et al.* 1958). The further use of this method was precluded by the finding that the extract contained glutamine, which is destroyed to a serious extent on such columns (Moore & Stein, 1954), while asparagine was present in such high concentration that it overlapped both aspartic acid and glutamine. A different method of fractionation was therefore developed to avoid these difficulties. The procedure is summarized in Fig. 1. In a multistage fractionation of this sort, care must be taken to avoid the accumulation of salts which are difficult to remove from the amino acid fractions after they are separated. In a similar process Hirs, Moore & Stein (1952) used buffers prepared from ammonium acetate, which was subsequently removed by vacuum sublimation. In a study involving ^{15}N it was thought desirable to avoid ammonium salts, and buffers based on calcium acetate were therefore used. Calcium is readily removed by precipitation with oxalic acid, and acetic acid by evaporation. This proved satisfactory except that calcium interfered with the separation of aspartic acid and arginine, presumably by the formation of an acidic complex with the arginine. This difficulty was easily overcome when it was recognized, but before this two samples containing a mixture of aspartic acid and arginine had been degraded to ammonia.

The first step in the fractionation procedure consisted in separating aspartic acid and glutamic acid from the other constituents of the extract, and from each other, by chromatography on a column of Amberlite IR-4B. The column (1 cm. × 80 cm.) was poured in calcium acetate buffer (Ca^{2+} 0·03 M, pH 4·5) and washed with the same buffer until the resin was in equilibrium with it. The extract (5 ml., pH 4·5) was then added and the column developed by gradient elution, that is with a solvent of constantly changing composition produced by running N-acetic acid into a reservoir (capacity 100 ml.) initially filled with the calcium acetate buffer with which the column had been prepared. The neutral and basic amino acids other than arginine passed straight through this column and their further separation is described below. This first peak was followed by glutamic acid and then by a third peak which was found to contain aspartic acid and arginine. These two amino acids were readily separated by chromatography on a short column of Zeo-Karb 225 at pH 5. The appropriate fractions were concentrated to 5 ml., and after adjusting the pH of the solution to 5 it was run on to the column of Zeo-Karb 225 (1 cm. × 7 cm.) previously equilibrated with disodium citrate (0·2 M, pH 5). The aspartic acid was washed off the column with a small volume of the same buffer and the arginine was then eluted with 2 M-disodium citrate.

The early fractions from the Amberlite column, containing the neutral and basic amino acids other than arginine, were mixed and freed from calcium by the addition of one

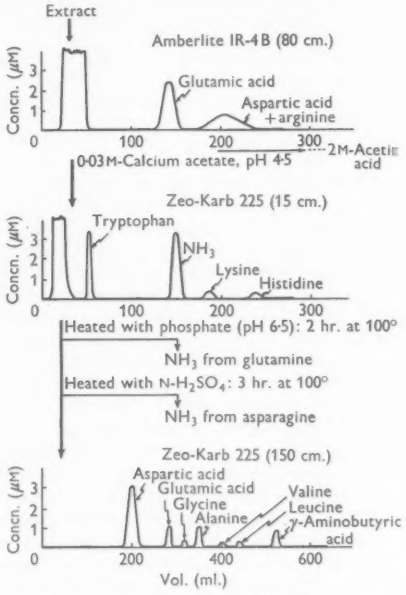


Fig. 1. Fractionation of soluble nitrogen of *Myrica* nodules.

equivalent of oxalic acid and centrifuging of the insoluble calcium oxalate. The supernatant was lyophilized and the residue taken up in 5 ml. of water. After adjustment of its pH to 5, this solution was placed on a short column of Zeo-Karb 225 (15 cm. × 1 cm.) prepared as described by Moore & Stein (1951). The column was developed with the sodium-containing buffers recommended by these authors and yielded tryptophan, free ammonia and basic amino acids well separated from one another.

The neutral amino acids, including asparagine and glutamine, were not retarded by this last column and passed through in a single band. The fractions containing them were transferred to a Van Slyke aeration tube in order to remove the amide groups from glutamine and asparagine. This was achieved by the method of Pucher, Vickery & Leavenworth (1935). Sodium hydroxide and phosphate buffer were added to give pH 6·5 and a phosphate concentration of 0·03 M, and the solution heated in a boiling-water bath for 2 hr. to decompose glutamine. The solution was then cooled, and adjusted to pH 9 with calcium hydroxide and the ammonia swept by aeration into boric acid (2%). The residual solution was acidified with sufficient sulphuric acid to make it N with respect to acid and the tube heated in a water bath for 3 hr. After cooling the solution was adjusted to pH 9 by the addition of calcium hydroxide and the ammonia from asparagine aerated into boric acid.

The mixture of amino acids now remaining in the reaction tube was separated by chromatography on a long column of Zeo-Karb 225 (150 cm. × 1 cm., Moore & Stein, 1954). Before addition to the column the solution was treated with oxalic acid until no further precipitation occurred, the precipitate of insoluble calcium salts was centrifuged off and the supernatant solution concentrated to 5 ml. and adjusted to pH 2·5.

The details of chromatographic technique used during these fractionations were similar to those described earlier (Leaf *et al.* 1958), as were also the methods of preparation for isotopic assay.

RESULTS

As indicated above, three experiments were carried out, each involving the exposure of nodules to excess of ^{15}N and their subsequent analysis.

The nodules contained some 0·7% of total nitrogen (fresh weight basis) of which 15–20% was soluble in dilute acid. The distribution of this soluble nitrogen among amino acids and ammonia is shown in Table 1. In the analysis of the extract from Expt. 1 a number of amino acids were lost during the final stage of the fractionation though they were known to be present as a result of paper chromatography of the original extract. In this experiment also, arginine was only partially separated from aspartic acid. Some arginine was obtained free from aspartic acid but not vice versa. Visual examination of the paper chromatograms of the original extract suggested that the major part of the nitrogen of the mixed fraction was contributed by the arginine. In Expt. 3 the mixed arginine–aspartic acid fraction was inadvertently degraded before separation of the two amino acids. Here also the greater part of the nitrogen was from arginine.

In Expt. 2, where the recovery of amino acids and ammonia was most complete, these constituents accounted for 63% of the extracted nitrogen. The nature of the remainder is unknown. There was close agreement between the ninhydrin determinations and the determination of total nitrogen by the Kjeldahl method, suggesting that undefined nitrogenous substances were present in the isolated fractions in amount insufficient to interfere with the isotopic analyses.

A sample of the residue, left after extraction of the soluble nitrogen, was hydrolysed with hydrochloric acid (15 hr. at 115°) and the hydrolysate examined qualitatively by paper chromatography. In addition to the seventeen amino acids commonly found in protein hydrolysates hydroxyproline was also found. Comparisons of paper chromatograms of the hydrolysate with controls prepared from a gelatin hydrolysate indicated that hydroxyproline accounted for some 2–4% of the insoluble protein of the nodules.

All the substances listed in Table 1 which were present in sufficient amount were assayed for ^{15}N and the results obtained are presented in Table 2. A high proportion of the ^{15}N taken up by the nodules was present in the extract. For Expt. 2 this proportion was 82% and for Expt. 3, where the exposure was shorter, 89%. Of the ^{15}N in the extract approximately 60% was recovered in the compounds analysed in Expts. 2 and 3. In all experiments the highest abundance of ^{15}N was found in the amide group of glutamine which

Table 1. *Amino acid composition of extracts of* Myrica gale *nodules*

Results are expressed as mg. of N/100 g. (fresh weight) of nodules. + indicates that the amino acid was shown to be present but the amount was not determined. Aspartic acid fractions in Expts. 1 and 3 were heavily contaminated with arginine. The experiments were carried out on 1, 2 and 9 August respectively.

	Expt. 1	Expt. 2	Expt. 3
Time of exposure to ^{15}N (min.)	120	60	45
Aspartic acid	25·9	3·5	12·6
Glutamic acid	6·2	6·4	5·6
Asparagine	18·9	27·5	18·7
Glutamine	2·9	2·8	2·6
Glycine	+	0·6	+
Alanine	0·5	1·5	1·3
Valine	+	0·4	0·4
γ-Aminobutyric acid	+	2·3	2·5
Tryptophan	2·3	3·0	6·6
Lysine	0·2	0·4	0·2
Arginine	+	3·9	+
Histidine	0·3	0·4	+
Ammonia	2·5	5·0	3·5

Table 2. *Distribution of* ^{15}N *in the constituents of the nodule extracts*

Results are given as atom % excess over the normal figure (0·375 atom %). The aspartic acid fractions in Expts. 1 and 3 were heavily contaminated with arginine.

	Expt. 1	Expt. 2	Expt. 3
Time of exposure to excess of ^{15}N (min.)	120	60	45
Total nitrogen of nodule	—	0·069	0·070
Extract nitrogen	—	0·334	0·372
Aspartic acid	0·186	0·427	0·049
Glutamic acid	1·087	0·602	0·595
Asparagine (amide N)	1·127	0·506	0·560
Asparagine (amino N)	0·328	0·149	0·196
Glutamine (amide N)	3·140	1·450	1·684
Glutamine (amino N)	0·630	0·460	0·261
Glycine	—	0·054	—
Alanine	0·270	0·810	0·155
Tryptophan	0·595	0·359	0·388
Arginine	0·018	0·002	—
Ammonia	0·876	0·736	0·540

showed a degree of enrichment quite striking for nodule tissue. The abundance of ^{15}N in the asparagine amide nitrogen was lower and approximately equal to that of the amino nitrogen of free glutamic acid. The enrichment of the amino nitrogen of the amides was in all cases less than that of the amide nitrogen and less than that of the corresponding free amino acid. The enrichment of the tryptophan nitrogen is remarkably high but it has not been possible as yet to determine how the ^{15}N is distributed between the two nitrogen atoms of the molecule.

DISCUSSION

Among the products of hydrolysis of the protein fraction of *Myrica* nodules was, as noted above, hydroxyproline. In animal tissues this latter amino acid occurs in certain structural proteins such as collagen. The observations of Steward and his collaborators suggest that, though the mechanism of formation of this amino acid is similar in plant and animal tissues (Steward & Pollard, 1958), its occurrence in the former is more restricted (Steward, Thompson & Pollard, 1958). Their findings of its abundant occurrence in the proteins of crown gall of *Kalanchoë* sp. and tobacco-leaf tumours is of interest here since it is not unreasonable to assume a relationship between such structures and a root nodule.

Of the amino acids found in the protein fraction only nine are represented among the free amino acids of the nodules (Table 1) and the concentrations of these are spread over a wide range. This finding of an unbalanced amino acid composition conforms to what is now a familiar pattern for extracts of plant tissues (e.g. see Steward & Pollard, 1956, 1957; Oland & Yemm, 1956; Bathurst, 1953, 1954*a*; Miettinen & Virtanen, 1952; Leaf *et al.* 1958). The reasons for the accumulation of certain amino acids in relatively high concentration are not known, but presumably reflect in some way metabolic idiosyncrasies of the cells concerned. This has suggested that nitrogen-fixing tissues such as root nodules might show distinctive features of amino acid composition. The available data do not substantiate this. Legume nodules were found to have a general similarity in this respect (Hunt, 1951; Sen & Burma, 1953; Bathurst, 1954*b*), but the work of Virtanen & Miettinen (1953) revealed dramatic differences between the nodules of pea and those of alder. The results of the present investigation show that though *Myrica*, like *Alnus*, is not a legume the pattern of amino acids in its nodules is more akin to those of the legume nodules than to that of *Alnus* nodules. The latter contain high concentrations of citrulline but no asparagine or glutamine (Miettinen & Virtanen, 1952; Leaf *et al.* 1958); *Myrica* nodules contain no citrulline but, like legume nodules, contain generous amounts of the amides. The nodules of *Myrica* differ from those of the pea, however, in that they do not contain detectable amounts of homoserine. It appears therefore that the amino acid composition of the nodule sap is determined by the nature of the host plant. This is what may be expected if the nitrogen fixed is presented to the plant in a form normally assimilated by plants without nodules.

It is generally assumed that the accumulations of amino acids and amides act as a reserve supply of nitrogen for the synthesis of protein and other essential substances. There is, however, little evidence to show how readily available are these reserves to the plant. In some instances such substances may accumulate because they are metabolically inert and, once formed, however slowly, remain unchanged. In this case their nitrogen would not be readily available to the plant.

The present findings with respect to the distribution of ^{15}N have some bearing on this point, for they show that with the exception of arginine all the substances present in appreciable amount incorporated this isotope quite rapidly. It is perhaps significant that arginine behaves exceptionally here, for the same was found in the work on *Alnus* (Leaf *et al.* 1958). Yemm & Folkes (1958) suggest that in certain plants, such as the pea and apple, arginine may serve a function similar to that generally ascribed to the amides, asparagine and glutamine. This appears to be unlikely in either *Alnus* or *Myrica*. It would therefore be interesting to determine the rate of turnover of the nitrogen of arginine in plants where it is a more conspicuous constituent of the soluble nitrogen.

The main object of the present investigation was to secure evidence on the mechanism of nitrogen

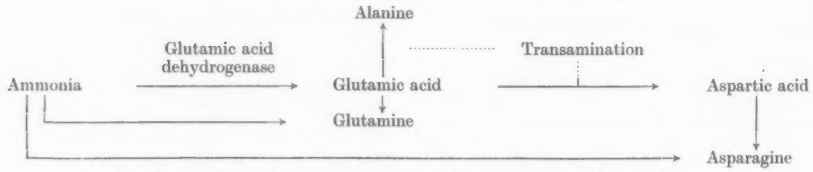


Fig. 2. Possible routes of assimilation of fixed nitrogen in *Myrica nodules*.

fixation in *Myrica* nodules. In examining the results in Table 2 from this point of view, it is notable that the nitrogen atoms showing the highest abundance of ^{15}N are, with the exception of those of tryptophan, all in chemical groups which might be expected on the basis of comparative biochemistry to be derived fairly directly from free ammonia (Fig. 2). Admittedly there is some uncertainty as to the origin of asparagine in plants. Webster & Varner (1955) studied the formation of this amide in lupin seedlings and concluded that it was formed by a reaction between ammonia and aspartic acid, catalysed by an enzyme similar to that involved in the synthesis of glutamine (Elliott, 1953). As pointed out by Steward & Pollard (1956), the concentrations of substrates required by this enzyme were, however, very much higher than those for the glutamine-synthesizing enzyme, so high indeed as to throw doubt on its physiological significance.

In *Myrica* nodules it appears that asparagine may be formed at least as rapidly as is glutamine. Though the abundance of ^{15}N is always greater in the amide group of glutamine than in that of asparagine, when account is taken of the relative amounts of the two amides present it becomes evident that the amount of ^{15}N incorporated into the amide group of asparagine is some $3\frac{1}{2}$ times that incorporated into the amide group of glutamine. This cannot be taken as a direct measure of the relative rates of synthesis, but it suggests that asparagine may be synthesized at least as rapidly as is glutamine. The data do not give any indication of the mechanism of asparagine synthesis, but would be compatible with either a more active mechanism of the type described by Webster & Varner (1955) or one involving transamidation from glutamine, such as was found in animal liver by Mardashev & Lestrovaya (1951). Whichever reaction sequence is involved the asparagine receives its nitrogen fairly directly from ammonia. Asparagine and glutamine have long been considered to act as repositories of nitrogen in excess of that required for immediate anabolic requirements. In *Myrica* nodules the amide groups of these compounds appear to act as traps into which the nitrogen, newly fixed and apparently in the form of ammonia, drains as it is formed.

The evidence of the distribution of ^{15}N in the amino acids thus strongly supports the view that ammonia is an obligatory intermediate between fixation and assimilation of nitrogen. Apparently in discord with this view is the observation that ammonia itself does not show a higher abundance of ^{15}N than any other fraction. This is a common finding in work of this type (see Leaf *et al.* 1958, for references), and is usually explained by the assumption that there are two pools of ammonia in the tissue, one receiving newly fixed nitrogen and the other inert in this respect. Inherent in this is a second assumption that the same effect does not obtain, at any rate to the same extent, with the amino acids and amides. This explanation receives support from the work of Allison & Burris (1957) with *Azotobacter* and such a situation is even more likely in a nodule in view of the greater structural complexity.

SUMMARY

1. Nodules of *Myrica gale* were exposed to excess of ^{15}N, after detachment from the plant, for periods of 45–120 min. The soluble nitrogen compounds were then extracted from the nodules and separated by chromatographic procedures.

2. The amino acid composition of the extract was thus determined and it was found that asparagine was the predominant amino acid. Glutamine and various other amino acids were present in smaller amounts.

3. The highest enrichment with ^{15}N was found in the amide N of glutamine and the next highest in the amide N of asparagine and amino N of glutamic acid.

4. The data are considered to support the view that the fixed nitrogen passes through the form of ammonia before entry into organic combination.

The authors are indebted to Dr R. H. Burris, of the University of Wisconsin, for his kindness in carrying out the ^{15}N assays reported in this paper. One of the authors (I.C.G.) held a Carnegie Research Scholarship during the period of the investigation.

REFERENCES

Allison, R. M. & Burris, R. H. (1957). *J. biol. Chem.* **224**, 351.

Bathurst, N. O. (1953). *J. Sci. Fd Agric.* **4**, 221.

Bathurst, N. O. (1954*a*). *J. exp. Bot.* **5**, 253.
Bathurst, N. O. (1954*b*). *J. exp. Bot.* **5**, 258.
Bond, G. (1951). *Ann. Bot., N.S.*, **15**, 447.
Bond, G. (1958). *Proc. 5th Easter Sch. Agric. Sci. Univ. Nottingham.*
Elliott, W. H. (1953). *J. biol. Chem.* **201**, 661.
Hirs, C. W. H., Moore, S. & Stein, W. H. (1952). *J. biol. Chem.* **195**, 669.
Hunt, G. E. (1951). *Amer. J. Bot.* **38**, 452.
Leaf, G., Gardner, I. C. & Bond, G. (1958). *J. exp. Bot.* **9**, 320.
Mardashev, S. R. & Lestrovaya, N. N. (1951). *Dok. Akad. Nauk. S.S.S.R.*, **78**, 547.
Miettinen, J. K. & Virtanen, A. I. (1952). *Physiol. Plant.* **5**, 540.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 893.
Oland, K. & Yemm, E. W. (1956). *Nature, Lond.*, **178**, 219.
Pucher, G. W., Vickery, H. B. & Leavenworth, C. S. (1935). *Industr. Engng Chem. (Anal.)*, **7**, 152.
Sen, S. P. & Burma, D. P. (1953). *Bot. Gaz.* **115**, 185.
Steward, F. C. & Pollard, J. K. (1956). In *Inorganic Nitrogen Metabolism*. Ed. by McElroy, W. D. & Glass, B. Baltimore: *The Johns Hopkins Press.*
Steward, F. C. & Pollard, J. K. (1957). *Annu. Rev. Pl. Physiol.* **8**, 65.
Steward, F. C. & Pollard, J. K. (1958). *Nature, Lond.*, **182**, 828.
Steward, F. C., Thompson, J. F. & Pollard, J. K. (1958). *J. exp. Bot.* **9**, 1.
Virtanen, A. I. & Miettinen, J. K. (1953). *Biochim. biophys. Acta*, **12**, 181.
Webster, C. G. & Varner, J. E. (1955). *J. biol. Chem.* **215**, 91.
Yemm, E. W. & Folkes, B. F. (1958). *Annu. Rev. Pl. Physiol.* **9**, 245.

Oxidative Enzyme Systems in Ossifying Cartilage

By R. G. WHITEHEAD and S. M. WEIDMANN
Biological Research Unit, School of Dentistry, University of Leeds

(*Received* 11 *December* 1958)

Previous studies into the metabolism of cartilage have been directed mainly towards the anaerobic phase of carbohydrate metabolism and little is known about the presence of aerobic enzyme systems in this tissue. Early work indicated that cartilage was almost anaerobic in nature, and Bywaters (1937) and Lutwak-Mann (1940) showed that in articular cartilage oxygen consumption was virtually absent. The latter author was also unable to demonstrate histochemically the presence of the cytochrome system, a finding which was subsequently confirmed by Follis & Berthrong (1948). Cartilage before ossification shows little vascularization and Cartier (1951*a*) stated that the resultant low oxygen concentration accounted for the relatively high concentration of lactic acid in cartilage. Dixon & Perkins (1952) demonstrated the presence of the condensing enzyme and aconitase in cartilage, but found that *iso*citric dehydrogenase activity was very low. This, it was claimed, explained the accumulation of citrate in hard tissues.

Hiatt & Shorr (1953) were, however, able to provide histochemical evidence for the existence of dehydrogenase systems in rachitic rat cartilage by using 2:3:5-triphenyltetrazolium chloride. Follis & Melanotte (1956) confirmed this finding, and in addition showed that the reduction of the dye was accelerated by certain intermediates of the Krebs cycle.

Various attempts to demonstrate manometrically the presence of dehydrogenase systems have been made and Boyd & Neuman (1954) demonstrated the presence of succinic dehydrogenase in embryonic chicken cartilage. Laskin & Engel (1956) confirmed this finding, but their results showed that glucose, pyruvate, citrate, α-oxoglutarate and fumarate did not affect the respiration of young metaphyseal bone slices. These results would indicate the absence of the Krebs cycle as an entity. The presence of succinic dehydrogenase has also been shown by Castellani & Zambotti (1954), who used a colorimetric procedure.

Recent work (Whitehead & Weidmann, 1959) on the effect of parathyroid extract on metabolism of adenosine triphosphate indicated the possible importance of oxidative phosphorylation in cartilage, but owing to the uncertainty in the literature as to the presence of an oxidative metabolism it was difficult to ascertain the significance of this suggestion. To clarify this situation, in the following work oxidative systems in ossifying cartilage were studied. The distribution of dehydrogenase and cytochrome oxidase systems was examined histochemically, and the nature of the various enzymes involved deduced from manometric studies. Oxidative phosphorylation in cartilage was also investigated by examining the effect *in vivo* of the injection of 2:4-dinitrophenol on the incorporation of ^{32}P into adenosine triphosphate.

METHODS

The material used in the present work was taken from the limb cartilages of young kittens aged 2–3 weeks.

Distribution of dehydrogenase activity. Slices about 0·2 mm. thick were cut, free-hand, longitudinally from the cartilage at the distal end of the femur and then placed in small flasks containing 10 ml. of Ringer phosphate (Umbreit, Burris & Stauffer, 1957) and 0·5% 2:3:5-triphenyltetrazolium chloride (Hiatt & Shorr, 1953). The vessels were equilibrated for 5 min. with either oxygen or nitrogen, and then incubated at 37° with occasional shaking for about 1 hr. The slices were examined immediately for red formazan deposition in the cells and then photographed. The colour fades quickly on exposure to light.

Distribution of cytochrome oxidase. The method used was the Moog (1943) modification of the Nadi reaction. Hand-cut slices similar to those described above were placed in the Nadi reagent, which was prepared by combining, immediately before use, equal parts of 0·01 M-*p*-amino-*NN*-dimethylaniline and 0·01 M-α-naphthol, each in 1% NaOH and 0·066 M-phosphate buffer (NaH_2PO_4 adjusted to pH 5·8 with NaOH). Control slices were first washed in aq. 0·09% NaCl containing 5 mM-sodium azide, then placed in a modified Nadi reagent also containing 5 mM-sodium azide. The low pH used was to facilitate the entry of azide into the cells. Moog (1943) showed that the uninhibited results were identical with those obtained at pH 7·2. The slices were incubated at 37° for about 30 min., then examined for indophenol-blue deposition in the cells and photographed.

Manometric studies. Longitudinal slices again about 0·2 mm. thick were cut from the limb cartilages and about 150 mg. (wet wt.) was transferred to a manometer flask, containing 2·8 ml. of Ringer phosphate (pH 7·4), and KOH in the centre well. Before equilibration of the flasks at 37° the system was flushed with oxygen. The various substrates used—citrate, α-oxoglutarate, succinate, malate, pyruvate (10^{-2} M) and glucose (5 mM)—were contained in 0·5 ml. of Ringer phosphate (pH 7·4) and added from the side arm after equilibration. The inhibitors, sodium arsenite (5 mM) and sodium malonate (10^{-2} M), were added in 0·1 ml. of Ringer phosphate directly to the main compartment of the flask. Readings were taken every 10 min. and the calculations for oxygen consumption based on the first hour of observation. On completion of the manometric measurements the weight of the cartilage sample was measured after drying to constant weight at 105°. The average amount was 20–30 mg.

Dinitrophenol injection. Litter-mate pairs of kittens were selected and the experimental kittens injected intraperitoneally with 20 mg. of 2:4-dinitriphenol in aq. 0·09% NaCl and the control animals with aq. 0·09% NaCl. Both groups were then returned to their mothers. Certain of the pairs were killed 24 hr. later; the remainder were given two subsequent daily injections of 2:4-dinitrophenol and killed 24 hr. after the final treatment. Two hours before death each animal was injected with 330 μc of carrier-free $H_3{}^{32}PO_4$/kg. body wt. Blood samples were taken under anaesthetic immediately before death, and the serum was analysed for inorganic phosphate and ^{32}P content as previously described (Rogers, Weidmann & Jones, 1953). The limb cartilages were analysed for adenosine triphosphate and orthophosphate, as recently reported (Whitehead & Weidmann, 1957). The units of specific activity are expressed as the ratio:

$$\frac{(\text{Counts/min./mg. of P}) \times 10^9}{\text{Administered counts/min./kg. body wt.}}.$$

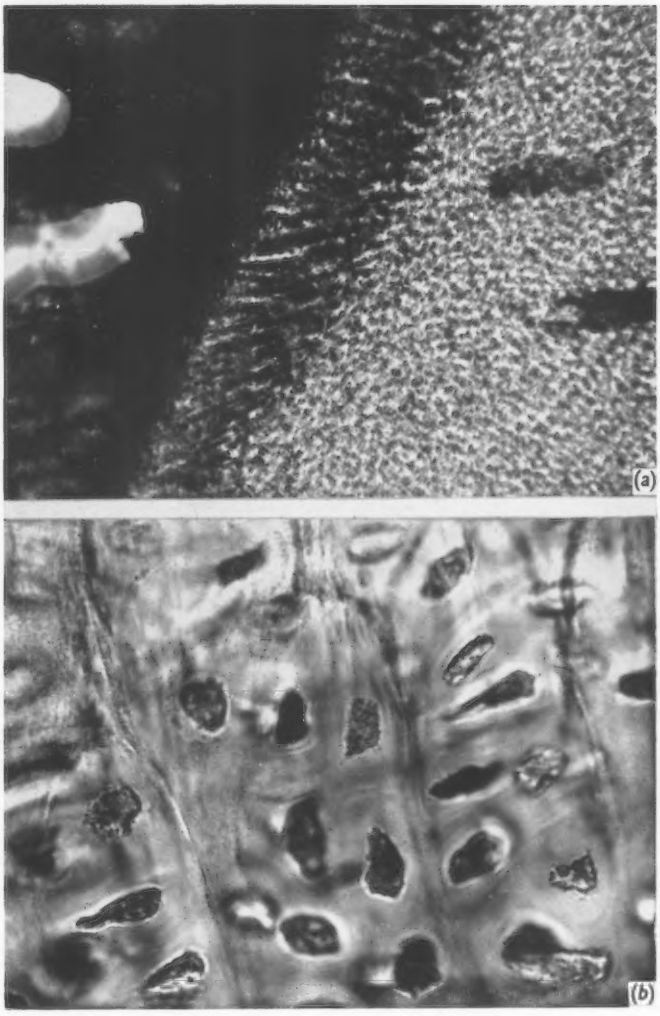
RESULTS

Distribution of dehydrogenase and cytochrome oxidase activity. In the histochemical studies, the reduction of 2:3:5-triphenyltetrazolium chloride produced deposition of red formazan only in those slices incubated in 100% nitrogen; those in oxygen remained colourless, thus indicating that the ultimate hydrogen acceptor is normally molecular oxygen. The formazan deposition, which indicates dehydrogenase activity (Mattson, Jenson & Dutcher 1947), occurred mainly in the hypertrophic zones of cartilage. In young kittens, aged about 7 days, the only definite red coloration was in the hypertrophic cells adjacent to the metaphysis; in older kittens when ossification centres have developed in the middle of the epiphyseal cartilage the hypertrophic cells in these areas also showed formazan deposition. In the remainder of the cartilage little or no red formazan was visible (Plate 3*a*).

The distribution of cytochrome oxidase was very similar to that of the dehydrogenases. Formation of granules of indophenol blue resulting from the oxidative coupling of α-naphthol and *p*-amino-*NN*-dimethylaniline, which according to Keilin (1933) indicates cytochrome oxidase activity, was apparent only in the hypertrophying cells (Plate 3*b*). In other areas no blue deposition could be seen. To confirm that the formation of indophenol blue was due to cytochrome oxidase activity, the effect of sodium azide was tested; this was observed to retard the reaction. From these two histochemical tests it would appear that the hypertrophic cells of cartilage are able to support an oxidative metabolism.

Manometric studies. To obtain more definite information about the oxidative systems in cartilage, the respiration of cartilage slices was investigated. Oxygen consumption, although low compared with that of liver slices from the same animals, was much greater than that reported by earlier workers. Using articular cartilage, Bywaters (1937) found Q_{O_2} values of 0·005 μl. of oxygen consumption/mg. dry wt./hr.; in the present work the corresponding values were about 100 times as much, i.e. between 0·5 and 0·9 for 2–3-week-old kittens. Boyd & Neuman (1954) found similar high values in embryonic chicken cartilage. Sodium cyanide (mM) almost completely inhibited the oxygen consumption of the cartilage slices.

To obtain information on the nature of the dehydrogenase systems and the possible operation of the Krebs cycle in cartilage, the stimulatory effect on respiration of various components of the cycle



(*a*) Band of hypertrophic cartilage cells, adjacent to the metaphysis, showing intense red-formazan deposition. This is indicative of dehydrogenase activity. (*b*) Hypertrophic cartilage cells showing indophenol-blue deposition within the cytoplasm. This is indicative of cytochrome oxidase activity.

R. G. WHITEHEAD AND S. M. WEIDMANN—OXIDATIVE ENZYME SYSTEMS IN OSSIFYING CARTILAGE

(*Facing p.* 668)

Table 1. *Effect of various substrates of the Krebs cycle on the respiration of cartilage slices*

All calculations for consumption are based on the first hour of observation.

Animal (no.)	Substrate	Endogenous respiration (μl. of O_2/mg. dry wt./hr.)	Respiration with substrate (μl. of O_2/mg. dry wt./hr.)
1	10^{-2}M-Citrate	0·17	0·64
2		0·19	0·69
3		0·50	0·90
4		0·37	0·92
5		0·62	1·60
6	10^{-2}M-α-Oxoglutarate	0·62	2·30
7		0·55	1·72
8		0·74	1·50
9	10^{-2}M-Succinate	0·86	1·51
10		0·71	1·50
11		0·60	1·36
12		0·50	1·10
13	10^{-2}M-Malate	0·50	0·90
14		0·60	1·71
15		0·60	1·40
16		0·56	2·06
17	10^{-2}M-Pyruvate	0·70	2·20
18		0·90	1·77
19		0·53	1·21
20		0·55	1·41
21	5 mM-Glucose	0·88	0·87
22		0·52	0·40
23		0·66	0·70
24		0·77	0·96

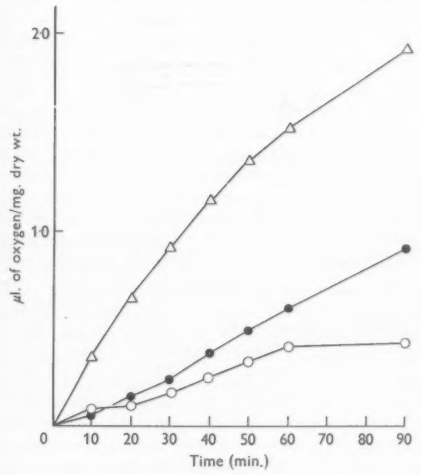


Fig. 1. Effect of arsenite on the oxidation of citrate by slices of epiphyseal cartilage taken from 9-day-old kittens. △, Respiration in the presence of 10^{-2}M-citrate; ○, respiration in the presence of 10^{-2}M-citrate and 5 mM-arsenite; ●, endogenous respiration.

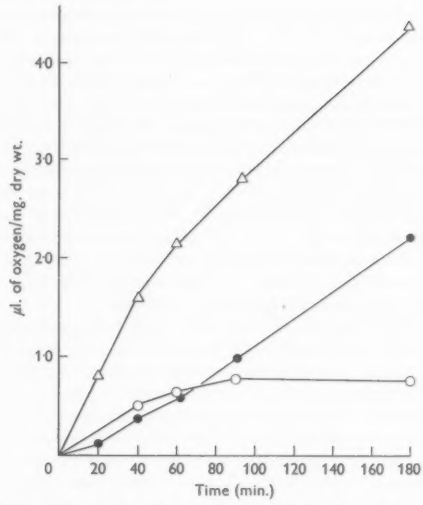


Fig. 2. Effect of arsenite on the respiration of α-oxoglutarate by slices of epiphyseal cartilage taken from a 10-day-old kitten. △, Respiration in the presence of 10^{-2}M-α-oxoglutarate; ○, respiration in the presence of 10^{-2}M-α-oxoglutarate and 5 mM-arsenite; ●, endogenous respiration.

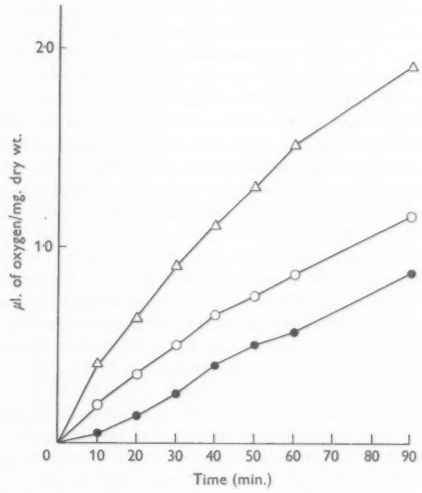


Fig. 3. Effect of malonate on the respiration of succinate by slices of epiphyseal cartilage taken from 9-day-old kittens. △, Respiration in the presence of 10^{-2}M-succinate; ○, respiration in the presence of 10^{-2}M-succinate and 10^{-2}M-malonate; ●, endogenous respiration.

was studied. Citrate, α-oxoglutarate, succinate and malate (all 10^{-2}M) significantly increased the oxygen consumption of the cells two- to three-fold over the first hour of measurement (Table 1). This indicated that *iso*citric dehydrogenase, α-oxoglutarate oxidative decarboxylase, succinic dehydrogenase and malic dehydrogenase were all present in cartilage. The stimulation of respiration was most prominent over the first $\frac{1}{2}$ hr. After this time the stimulatory effect became less pronounced and ceased after about $1\frac{1}{2}$ hr. (Figs. 1–3). Pyruvate (10^{-2}M) produced a similar stimulation of respiration, but 5 mM-glucose had no significant effect on the oxygen consumption of cartilage.

The actions of the two enzymic inhibitors, sodium malonate (10^{-2}M) and sodium arsenite (5 mM), on the stimulated respiration produced by the various substrates were tested to verify the presence of succinic dehydrogenase and α-oxoglutarate oxidative decarboxylase. Sodium arsenite caused a very marked inhibition of oxygen consumption both with citrate as substrate (Fig. 1) and with α-oxoglutarate (Fig. 2). Sodium malonate produced a partial inhibition with succinate (Fig. 3).

Effect of 2:4-dinitrophenol on the incorporation of ^{32}P into cartilage orthophosphate and adenosine triphosphate. The injection of 2:4-dinitrophenol did not appreciably alter the growth rate of the animals and they continued to feed quite normally from the mother. Neither single nor multiple injection of 2:4-dinitrophenol had any significant effect on the specific activity of ^{32}P contained in the serum of experimental animals ($201\,000 \pm 9\,000$) compared with the controls ($201\,000 \pm 9\,000$). The results are expressed as mean values ± S.E.M. and are calculated for the 9 pairs of animals. The corresponding values for serum inorganic phosphate were similarly unaffected ($8{\cdot}6 \pm 0{\cdot}4$; $8{\cdot}8 \pm 0{\cdot}5$). Consequently the amount of isotope available at the tissue sites was unchanged by the treatment.

Injection of 2:4-dinitrophenol resulted in a very significant reduction in the incorporation of ^{32}P into cartilage adenosine triphosphate measured after 24 hr. (Table 2), and this was maintained by multiple injection. On the other hand, the cartilage orthophosphate showed no significant difference in the specific activity of incorporated ^{32}P between the experimental and control animals after the single injection (Table 3). After repeated injection of the dinitrophenol, however, a significant diminution of isotope incorporation was observed also in the orthophosphate. These results are very similar to those obtained with parathyroid extract (Whitehead & Weidmann, 1959), where again only multiple injections of the hormone affected the orthophosphate specific activity; the adenosine triphosphate showed a significantly reduced turnover after the single injection.

DISCUSSION

Cartilage is poorly vascularized, resulting in a low concentration of oxygen in the cells. It possesses a high rate of anaerobic glycolysis (e.g. Roche & Deltour, 1943; Gutman & Yü, 1949). These facts, together with the importance attached to alkaline phosphatase and the hydrolysis of glycolytic phosphate esters in calcification (Robison, 1923), have resulted in attention being centred almost completely on anaerobic metabolism. Most of the work on aerobic metabolism was done with non-calcifying articular cartilage, and the conclusions reached from this tissue indicated that the aerobic metabolism of cartilage was almost negligible.

The results in the present paper provide definite evidence for the existence of an oxidative metabolism in actively ossifying cartilage. The histochemical distribution of dehydrogenase and cytochrome oxidase activity indicates that this is

Table 2. *Effect of 2:4-dinitrophenol on the specific activity of adenosine triphosphate in cartilage measured 2 hr. after the injection of ^{32}P*

Litter no.	Specific activity: Experimental	Specific activity: Control	Effect (%)
	Multiple injections		
1	18 500	32 600	−43
2	18 500	32 600	−43
3	13 500	24 800	−46
4	16 000	29 000	−45
Average			−44*
	Single injections		
5	10 700	22 600	−53
6	16 700	25 000	−34
7	13 500	28 000	−52
8	19 000	33 000	−42
9	15 000	30 000	−48
Average			−44†

* Significant at $P < 0{\cdot}01$.
† Significant at $P < 0{\cdot}001$.

Table 3. *Effect of 2:4-dinitrophenol on the specific activity of orthophosphate in the cartilage epiphyses of kittens measured 2 hr. after the injection of ^{32}P*

Litter no.	Specific activity: Experimental	Specific activity: Control	Effect (%)
	Multiple injections		
1	51 400	73 500	−31
2	42 000	73 500	−43
3	27 400	44 400	−38
4	40 000	65 000	−40
Average			−38*
	Single injections		
5	62 200	60 400	+3
6	44 000	44 800	−2
7	68 000	67 500	+1
8	72 000	74 000	−3
9	79 100	78 800	0

* Significant at $P < 0{\cdot}01$.

particularly true in the hypertrophic cartilage cells. These areas have a rich blood supply and, presumably, the oxygen concentration is relatively high in the cells. That cells away from this area show no dehydrogenase or cytochrome oxidase activity indicates a low concentration of oxygen, which would be expected from the relatively avascular nature of the tissue. It is the hypertrophic region which subsequently undergoes calcification, and these ossifying regions are equipped with an active oxidative-enzyme system. This finding of a preferential distribution of dehydrogenases in the calcifying region differs from the histochemical results of Hiatt & Shorr (1953) and Follis & Melanotte (1956), who found the activity distributed evenly throughout rachitic-rat cartilage. According to the latter authors, as a consequence of their even distribution the oxidative enzymes would appear to have no bearing on the calcification mechanism. This statement requires re-examination in the light of the present results. Castellani & Zambotti (1954) found the concentration of succinic dehydrogenase highest in the layer of hypertrophic cells, and subsequently Castellani & Bianco (1956) showed that its activity was much higher in calcifying metaphyseal cartilage than in costal cartilage. Aerobic metabolism is more favourable than anaerobic metabolism for the synthesis of adenosine triphosphate, and Cartier (1951*b*) has demonstrated the probable importance of this compound in endochondral calcification. Cartier & Picard (1955) have shown, in addition, that the concentration of this compound is particularly high in the mineralizing zone.

The preferential distribution of oxidative enzymes in the narrow hypertrophic region may possibly explain the relatively low respiration of cartilage slices. It is probable that the majority of the respiration measured results from this area and the true oxygen consumption values should be attributed only to the hypertrophic cells. If this were the case then the corrected values would be substantially higher than those reported and possibly comparable with those of the liver.

The manometric investigations indicated the presence in cartilage of *iso*citric dehydrogenase, α-oxoglutarate oxidative decarboxylase, succinic dehydrogenase and malic dehydrogenase. Previously only succinic dehydrogenase has been demonstrated with certainty, and the results of Laskin & Engel (1956) indicated that it was the only dehydrogenase present. Since citrate was used as the substrate to show *iso*citric dehydrogenase activity this provides indirect evidence for the presence of aconitase. Pyruvate also stimulated respiration and this demonstrates that the condensing enzyme is possibly present in cartilage. Sodium arsenite inhibited oxygen consumption both with citrate and α-oxoglutarate as substrate, and since arsenite inhibits the oxidative decarboxylation of α-oxoglutarate to succinate it can be said with certainty that the portion of the Krebs cycle between citrate and succinate is present in entirety in cartilage cells. The effect of malonate also confirms the existence of succinic dehydrogenase, and since malate and pyruvate are oxidized by cartilage the most likely inference is that the whole of the Krebs cycle is operative in hypertrophic cartilage cells.

To couple the Krebs cycle with oxidative phosphorylation involves the cytochromes and cytochrome oxidase, but their presence in cartilage cells has previously been denied (Lutwak-Mann, 1940; Follis & Berthrong, 1948). The present results, however, revise the position. The existence of cytochrome oxidase has been demonstrated by a positive Nadi reaction in the hypertrophic cells. The results with 2:3:5-triphenyltetrazolium chloride are also indicative of the presence of cytochromes since the reduction potential of the dye is in the same range as the cytochromes with which it interacts in the reduction to red formazan. The cyanide sensitivity of the respiration provides confirmatory evidence for the presence of the cytochrome system in cartilage.

It is generally assumed that 2:4-dinitrophenol uncouples oxidative phosphorylation. If this is true, the present studies *in vivo* with dinitrophenol on the uptake of ^{32}P into adenosine triphosphate indicate the possible existence of oxidative phosphorylation in cartilage cells.

Stoner, Threlfall & Green (1952) and Stoner & Threlfall (1958), who studied the effect of inhibitors of oxidative phosphorylation in brain and skeletal muscle metabolism *in vivo*, were unable to show a decreased incorporation of ^{32}P into adenosine triphosphate, even when the dosage was so great that the animal was at the brink of death. These results were explained by suggesting that isotopic incorporation is normally so rapid that a partial inhibition of the process would not be apparent. This is apparently not true of cartilage.

As was observed with a preparation of parathyroid hormone (Whitehead & Weidmann, 1959), dinitrophenol affects orthophosphate specific activity only after a period of two or three daily injections, although the adenosine triphosphate turnover is significantly reduced after only one injection. It has been suggested that adenosine triphosphate is responsible for the initiation of calcification by the formation of a primary seed, which then stimulates the deposition of bone salt independently of cellular metabolism (Cartier & Picard, 1955); the relationship found between the ^{32}P incorporated into cartilage bone salt and adenosine triphosphate is in agreement with this

hypothesis. To explain the present results, it is suggested that there is normally an excess of seed present; consequently at short time-intervals after the injection of 2:4-dinitrophenol no significant reduction in bone-salt deposition is apparent. The reduced seed formation caused by the diminished synthesis of adenosine triphosphate does become manifest eventually and a reduced bone-salt deposition results. This is accompanied by a decrease in the specific activity of the orthophosphate owing to the presence of less of the newly formed bone salt available for ionic exchange.

Although previous studies of the calcification of cartilage were concerned almost exclusively with glycolysis, certain results were difficult to explain on the grounds of complete anaerobiosis. Gutman & Yü (1949) showed that 2:4-dinitrophenol (10^{-4} M) and gramicidin markedly inhibited calcification *in vitro*. Although it was stated that these inhibitors were known to dissociate inorganic phosphate uptake from hydrogen transfer in oxidative phosphorylation, it was difficult to see the importance of this relationship in the calcification of cartilage owing to its anaerobic nature. Several workers have shown that the calcification of cartilage slices *in vitro* is cyanide-sensitive; although this would indicate the importance of the cytochrome system in the calcification of cartilage, Goldenberg & Sobel (1951) state that the absence of this system in cartilage rules out the role of cyanide as an inhibitor of oxidative systems. No alternative suggestion could be made. Although adenosine triphosphate has been shown to be of great importance in calcification *in vitro*, Picard & Cartier (1956) have said that the high glycolytic activity compared with the low respiratory activity means the former system must provide the necessary adenosine triphosphate. The present results have shown that, whereas in the non-calcifying areas of cartilage the metabolism appears to be anaerobic, the region of cartilage which is undergoing rapid calcification is equipped with all the systems required for an active aerobic metabolism. On these grounds the previous studies *in vitro* require reinterpretation.

SUMMARY

1. Calcifying cartilage possesses both dehydrogenase and cytochrome oxidase systems, present predominantly in the hypertrophic cells.

2. Ossifying cartilage shows a much greater respiratory activity than has been reported for non-ossifying articular cartilage. The respiration is cyanide-sensitive.

3. The respiration of cartilage was stimulated by citrate, α-oxoglutarate, succinate, malate and pyruvate. It was inhibited by arsenite in the presence of citrate or α-oxoglutarate and by malonate in the presence of succinate.

4. The synthesis of adenosine triphosphate *in vivo*, measured by incorporation of ^{32}P, is inhibited by 2:4-dinitrophenol; uptake of isotope into the bone salt was inhibited only after longer treatment with dinitrophenol.

5. The possible significance of oxidative phosphorylation in calcification has been discussed.

Thanks are due to Mr L. Jepson for the photography. This work has been supported by a grant from the Medical Research Council.

REFERENCES

Boyd, E. S. & Neuman, W. F. (1954). *Arch. Biochem. Biophys.* **51**, 475.
Bywaters, E. G. L. (1937). *J. Path. Bact.* **44**, 247.
Cartier, P. (1951*a*). *C.R. Soc. Biol., Paris*, **145**, 274.
Cartier, P. (1951*b*). *Bull. Soc. Chim. biol., Paris*, **33**, 161.
Cartier, P. & Picard, J. (1955). *Bull. Soc. Chim. biol., Paris*, **37**, 1169.
Castellani, A. A. & Bianco, L. (1956). *R.C. Ist. lombardo.* **90**, 302.
Castellani, A. A. & Zambotti, V. (1954). *Boll. Soc. ital. Biol. spar.* **30**, 453.
Dixon, T. F. & Perkins, H. R. (1952). *Biochem. J.* **52**, 260.
Follis, R. H. & Berthrong, M. (1948). *Amer. J. Path.* **24**, 685.
Follis, R. H. & Melanotte, P. H. (1956). *Proc. Soc. exp. Biol., N.Y.*, **93**, 382.
Goldenberg, H. & Sobel, A. E. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 719.
Gutman, A. B. & Yü, T. F. (1949). *Trans. 1st Macy Conf. Metabolic Interrelations*, **1**, 11.
Hiatt, H. H. & Shorr, E. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 543.
Keilin, D. (1933). *Ergebn. Enzymforsch.* **2**, 239.
Laskin, D. M. & Engel, M. B. (1956). *Arch. Path.* **62**, 296.
Lutwak-Mann, C. (1940). *Biochem. J.* **34**, 517.
Mattson, A. M., Jenson, C. D. & Dutcher, R. A. (1947). *Science*, **106**, 294.
Moog, F. (1943). *J. cell. comp. Physiol.* **22**, 223.
Picard, J. & Cartier, P. (1956). *Bull. Soc. Chim. biol., Paris*, **38**, 697.
Robison, R. (1923). *Biochem. J.* **17**, 286.
Roche, J. & Deltour, G. H. (1943). *Bull. Soc. Chim. biol., Paris*, **25**, 1261.
Rogers, H. J., Weidmann, S. M. & Jones, H. G. (1953). *Biochem. J.* **54**, 37.
Stoner, H. B. & Threlfall, C. J. (1958). *Biochem. J.* **69**, 376.
Stoner, H. B., Threlfall, C. J. & Green, H. N. (1952). *Brit. J. exp. Path.* **33**, 398.
Umbreit, W. W., Burris, R. H. & Stauffer, J. H. (1957). *Manometric Techniques*, p. 149. Burgess Publishing Co. Minneapolis.
Whitehead, R. G. & Weidmann, S. M. (1957). *Nature, Lond.*, **180**, 1196.
Whitehead, R. G. & Weidmann, S. M. (1959). *Biochem. J* **71**, 312.

Selective Accumulation of Tritium-Labelled Hexoestrol by the Reproductive Organs of Immature Female Goats and Sheep

By R. F. GLASCOCK and W. G. HOEKSTRA*
The National Institute for Research in Dairying, University of Reading, Shinfield, Berks

(*Received* 14 *January* 1959)

A considerable amount of work has been done on the metabolism by living animals of oestrogens, both natural and synthetic, but, as pointed out by Dodds, Folley, Glascock & Lawson (1958), the doses given have nearly always been very large compared with the physiological dose. This has been necessary because the methods available for the detection of the oestrogens or their derivatives were relatively insensitive even when labelling with ^{14}C was used (Twombly, 1951; Twombly & Schoenewaldt, 1951; Hanahan, Daskalakis, Edwards & Dauben, 1953; Budy, 1955). Apart from the work of Dodds *et al.* (1958) (see below) the smallest doses have been used by Hanahan *et al.*, who gave [^{14}C]-stilboestrol to rats in doses of 5 μg., which is still more than ten times the physiological dose. It has been generally concluded that these oestrogens do not accumulate in organs such as uterus, vagina, mammary gland and pituitary gland, which respond to them physiologically. If this is so it would be necessary to attribute the response of these organs to some such mechanism as a selective sensitivity to the circulating oestrogen.

Glascock (1956) pointed out that by using tritium as a labelling agent very high specific activities are attainable and that this is extremely important when substances biologically as potent as the oestrogens are studied. In the rat, for example, 0·2 μg. of hexoestrol administered subcutaneously has a detectable action on the vagina (Dodds *et al.* 1944), whereas about $\frac{1}{2000}$ of the subcutaneous dose is effective when administered locally to the vagina (Emmens, 1940–41). This indicates that perhaps only a very small portion of a subcutaneous dose of oestrogen need reach the responding organs and that extremely sensitive methods of detection, and the use of the smallest effective doses, are probably necessary if any selective localization of the oestrogen in these organs is to be demonstrated.

Dodds *et al.* (1958) traced the excretion of microgram doses of tritium-labelled hexoestrol (specific activity about 10^7 counts/min./μg.) in rats and rabbits, but did not measure the radioactivity of body tissues. Subsequent studies with rats (M. Gabr & R. F. Glascock, unpublished work) indicated that a fleeting accumulation of hexoestrol, which was maximal about 7 hr. after a subcutaneous injection in oil, occurred in uterus, liver and, to a lesser extent, in kidney.

The purpose of the present study was to follow the distribution in organs and excreta of small doses of labelled hexoestrol at various times after injection into immature female goats and sheep. Distribution studies in ruminants were of additional interest because of the use of synthetic oestrogens in livestock production to increase rate of gain in body weight. By using physiological doses of hexoestrol it has been possible to show a selective distribution in those organs most sensitive to oestrogens as well as in those organs involved in their excretion. A preliminary account of some of this work has been published (Glascock & Hoekstra, 1958).

METHODS

Tritium-labelled hexoestrol. This was prepared by hydrogenation of dienoestrol with tritium–hydrogen on the micro scale over a palladium catalyst, after which it was purified and separated chromatographically from the less oestrogenically potent isomer *iso*hexoestrol (R. F. Glascock & G. S. Pope, unpublished work; for a preliminary account of the method see Glascock, 1954*a*). The hexoestrol is believed to be generally labelled in stable positions. The labelled hexoestrol was stored in the dark as a dilute solution in benzene (283 μg./ml.) to minimize radiolysis. A suitable portion of the benzene solution was placed in a small flask, the benzene removed under a stream of nitrogen and the labelled hexoestrol dissolved in arachis oil to give a concentration of 50 μg. of hexoestrol/ml. This solution was injected subcutaneously into the experimental animals. The original benzene solution of hexoestrol was assayed for radioactivity by the butane gas method of Glascock (1954*b*) after suitable dilutions with unlabelled hexoestrol. The arachis oil solution of hexoestrol was also assayed for radioactivity to verify the assay result and to make sure that the oily solution used for injection had been thoroughly mixed. The tritium-labelled hexoestrol was shown by both assays to have a specific activity of $0{\cdot}97 \times 10^7$ counts/min./μg.

Treatment of experimental animals. In the experiment with kids five immature crossbred females were used. They were 87–100 days old and weighed 14·3–22·8 kg. On separate days each kid received a subcutaneous injection (at a site on the left side of the neck) of 25 μg. of tritium-labelled hexoestrol contained in 0·5 ml. of arachis oil. This is about

* Present address: Department of Biochemistry, University of Wisconsin, Madison 6, Wisconsin, U.S.A.

the physiological dose of hexoestrol (see Discussion). Each kid was maintained in a metabolism cage for separate quantitative collection of urine and faeces. The kids were killed at 2, 5·5, 8, 24 and 48 hr. after the injection by cutting the blood vessels of the neck and bleeding to death under pentabarbitone sodium anaesthesia. Blood samples were taken from the last three animals at approximately the same intervals until death. The various organs and tissues were removed promptly with care to prevent any contamination with bile, urine or intestinal contents. Blood was rinsed from the surface of each organ, but no attempt was made to remove residual circulating blood.

A similar experiment was done on lambs to confirm the results of the experiment on kids and to extend observations on the distribution of hexoestrol to certain additional organs. Four crossbred female lambs about 4 months old and weighing 23·9–30·4 kg. were used. Each of the first two lambs received a single subcutaneous injection of 24 μg. of tritium-labelled hexoestrol in 0·48 ml. of arachis oil. One was killed 4 hr. and the other 8 hr. afterwards. Each of the other two lambs received the same total dose by eight injections at hourly intervals of 3 μg. of hexoestrol in 0·06 ml. of arachis oil. They were killed 8 hr. after the first injection. The divided dose was given in an attempt to simulate physiological conditions more nearly, since the previous experiment with kids had demonstrated a rapid absorption of hexoestrol from the injection site. Blood samples were taken at 20 or 30 min. intervals up to the time of killing.

Preparation of excreta and tissues for assay of radioactivity

In both experiments, faeces, intestinal contents (including abomasum contents), bile (in gall bladder), urine (passed) and urine (in bladder) were assayed separately. Faeces and intestinal contents were weighed and thoroughly mixed in a Waring Blendor with known amounts of water. A small portion representing 1–2 g. of dry matter was removed and weighed, and 1–2 mg. of unlabelled hexoestrol was added as carrier to trap the small quantities of radioactive hexoestrol which might otherwise be lost by volatilization. The sample was freeze-dried under vacuum, weighed and assayed for radioactivity. Urine and bile samples were freeze-dried after addition of carrier hexoestrol and assayed. To avoid inconveniently high counting rates it was necessary to dilute some of the samples of bile and urine solids before assay by adding a known weight of dextrin to the measured samples before freeze-drying.

The organs and tissue collected from the kids for assay of tritium were: blood, uterus, vagina, mammary glands, ovaries, pituitary gland, liver, kidneys, lungs, intestine, skeletal muscle (thigh), perinephric fat, pancreas, adrenal glands, salivary glands and skin. The discrete organs were weighed. Most of the organs were cut into small pieces (or minced in a domestic mixer) and thoroughly disrupted and mixed with about 2 vol. of water in a Waring Blendor or small laboratory homogenizer (depending on sample size) and the mince was strained through muslin to remove any uncomminuted connective tissue; finally a representative portion was stored in the deep-freeze. For assay the samples were thawed, a portion of about 10 ml. of homogenate was taken, 1–2 mg. of carrier hexoestrol was added and the sample was freeze-dried to provide the dried sample for combustion and assay of tritium. Blood samples were freeze-dried directly after addition of carrier. The uteri were divided into the endometrium and the myometrium for separate assay. This was done as well as possible by scraping the inner surface of the uterus with a microtome blade, but the separation was not complete and considerable endometrial tissue was included with the myometrium. Fat was melted in an oven. The skin samples from the abdomen, which could not be prepared in the manner described, were digested in 10% (w/v) NaOH in aqueous 50% ethanol for 24 hr. on a steam bath. The digest was then acidified to pH 2–3 and extracted five times with diethyl ether; the ether extract was dried with anhydrous Na_2SO_4, the solvent was removed and the extract assayed. Radioactivity was calculated per unit original dry weight. Thus for skin only those metabolites of hexoestrol which were soluble in diethyl ether after alkaline hydrolysis were measured. The relationship of this amount to the total is not known, but it is believed that the major portion of the radioactivity was extracted by this process.

The experiment with lambs was similar except that samples of oviducts, brain, mesenteric lymph nodes, thymus, heart, spleen, and bone (femur) were also taken, and samples of salivary glands and pancreas were not. The site of injection was also assayed. The femur was cut and the marrow and spongy bone scraped free with a knife and treated as the other tissues. Sections of the bone shaft were frozen in liquid air, crushed in a cooled block-and-piston arrangement, passed several times through a mincer, dried and assayed. The injection site included nearby skin and muscle and weighed 150–200 g. (fresh wt.). These tissues were cut into small pieces and digested in excess of ethanolic NaOH for 48 hr. on a steam bath. Measured portions of the digest were neutralized, a weighed amount of dextrin was added to dilute the radioactivity of the dry matter, carrier hexoestrol was added and the samples were freeze-dried, weighed and assayed.

Measurement of radioactivity. The water obtained by combustion of 15–30 mg. of dried samples was converted into butane and counted in a gas counter as described by Glascock (1954*b*). The yields of combustion water, required for calculation of specific activities of the dry tissue itself, were ascertained by condensing the combustion water into a small tube under high vacuum. The tube was sealed and removed from the high-vacuum line, marked with a glass knife and weighed. The weighed tube was broken at the mark, returned to a chamber on the high-vacuum line, frozen with liquid air and evacuated. The water was transferred to the tube containing butylmagnesium bromide for conversion into butane, and the empty tube which had contained water was removed from the vacuum line and weighed. This procedure was shown to be reliable by subjecting pure compounds (glucose and stearic acid), of known water yield and radioactivity, to combustion. Butane samples of total activity of several hundred counts/min. were counted for 10 000 counts or more, whereas fewer counts were made on samples of lower total activity down to a total of not less than 1000 counts for total activities of the same order as background (30 counts/min.). The combined accuracy of water-determination and counting of the samples with higher activity appeared to be within $\pm 3\%$ and was somewhat worse for those with lower activities. Nevertheless, specific activities as low as 2 counts/min./mg. dry matter could be reproducibly

Table 1. *Gross distribution of hexoestrol in female kids and lambs*

The kids each received a single subcutaneous injection of 25 μg. of tritium-labelled hexoestrol in arachis oil and were killed at the indicated times afterwards. Lambs 1 and 2 each received a single subcutaneous injection of 24 μg. of hexoestrol and were killed 4 and 8 hr. later. Lambs 3 and 4 each received eight injections of 3 μg. at intervals of 1 hr. and were killed 8 hr. after the first injection.

	Estimated percentage of total dose in sample								
	Kids					Lambs			
Animal no.	1	2	3	4	5	1	2	3	4
Body wt. (kg.)	14·3	17·7	22·8	14·0	14·2	30·4	25·0	23·9	25·9
Time after injection (hr.) ...	2	5·5	8	24	48	4	8	8	8
Dose	Single	Single	Single	Single	Single	Single	Single	Divided	Divided
Site of injection (skin and muscle)	—	—	—	—	—	21·6	1·8	7·1	12·8
Other body tissues*	28·7	20·7	10·5	1·5	0·8	30·7	16·6	26·3	26·7
Bile	1·3	5·4	3·9	0·4	0·04	4·1	0·6	2·0	3·0
Intestinal contents	7·8	31·8	40·9	17·4	1·1	18·0	49·8	43·8	36·2
Faeces	0·0	1·0	0·5	18·4	36·1	0·04	0·07	0·2	0·0
Urine	9·0	26·5	29·8	43·2	39·8	6·7	29·3	14·1	13·8
Total	56·8	85·4	85·6	81·0	77·8	81·1	98·2	93·5	92·5
Total hexoestrol in urine/total hexoestrol in bile, intestinal contents and faeces	0·47	0·69	0·66	1·19	1·07	0·31	0·58	0·31	0·35

* See Table 4.

detected. For the assay of such samples any contamination of the vacuum line by a previous sample was allowed for by applying a correction derived from the assay of unlabelled glucose. The correction provided by this blank was always less than 1 count/min./mg. dry matter. Samples of organs and tissues counted at rates from 0 to 205 counts/min./mg. dry matter (0–350 counts/min./mg. of combustion water), whereas those from certain of the excretory products, some of which were diluted with dextrin, counted at considerably higher rates (up to 700 counts/min./mg. dry matter).

RESULTS

In the calculations the radioactive compound in the samples is assumed to be hexoestrol itself and will be referred to as such, although in fact its identity has not yet been established.

General distribution of hexoestrol. In Table 1 is shown the gross distribution of hexoestrol (expressed as a percentage of the dose) at intervals after its injection into the kids and lambs. An exact radioactivity balance could not be made because the total weight of every organ assayed (e.g. muscle, skin, intestine) was not known, and the total radioactivities of the residual carcasses were not measured. However, an approximate measure of total radioactivity in the tissues was made from dry-matter content and their estimated contribution to the total body weight (muscle 40 %, bone and skull 20 %, skin 10 %, intestine 2 %). Total recoveries of injected radioactivity thus calculated for the kids (Table 1) were 56·8 % from the kid which was killed 2 hr. after injection and 79–86 % for the other four killed 5·5–48 hr. after injection. The low recovery for the first animal would be consistent with incomplete absorption of the hexoestrol from the injection site, which was not assayed in this experiment. However, even after 24 and 48 hr., when tissue levels of radioactivity were very low and nearly all the injected radioactivity was in urine, faeces and intestinal contents, total recoveries were only about 80 %. The reason for incomplete recoveries is not known. All samples of body water, as represented by the water obtained from blood samples during freeze-drying, had less than 1 count/min./mg., indicating little or no complete oxidation of hexoestrol (a counting rate of 1 count/min./mg. of body water would be equal to about 4 % of the total dose). Also no detectable radioactivity (<1 count/min./mg. of water) followed the volatile fraction during freeze-drying of urine and faeces. In the experiment with lambs, in which the sites of injection were assayed and all lambs were killed within 8 hr. of the injection, total recoveries were 81, 98, 94 and 92 %. Twombly & Schoenewaldt (1951) reported that dried tissues from animals injected with stilboestrol labelled with ^{14}C in the β-ethyl position lost radioactivity even when stored in the refrigerator. In this work the samples from the lambs were in general counted after shorter periods of storage than those from the kids but it is not known whether or not this is significant.

It is evident (Table 1) that hexoestrol injected in arachis oil was quite rapidly absorbed from the site of injection. Thus after 2 hr. (kid 1) at least 57 % and after 5·5 hr. (kid 2) at least 85 % of the dose was distributed in the body tissues and excretory pathways. It was shown by direct assay

that in the lambs (Table 1) 21·6 % of the injected hexoestrol remained at the injection site 4 hr. after a single dose whereas only 1·8 % was found there after 8 hr.

The injected hexoestrol rapidly passed into excretory pathways (urine, bile and intestinal contents). The percentages of the total dose in these pathways were 28, 65 and 75 % respectively in kids killed 2, 5·5 and 8 hr. after injection and 29 and 80 % in lambs killed 4 and 8 hr. after injection. One route of excretion was the bile, in which as much as 5·4 % of the dose was found 5·5 hr. after injection. The radioactivity from the bile undoubtedly contributed the major portion, if not all, of the radioactivity found in the intestinal contents. The urine was the only other major route of hexoestrol excretion. Table 1 also gives the ratios of the amounts of radioactivity in urine (including bladder urine) to the combined amount in bile, intestinal contents and faeces. With increasing time after injection of the labelled oestrogen a greater relative portion of the radioactivity was found in the urine.

Consistent with the rapid excretion of hexoestrol was a rapid decrease in the amount found in the body tissues: 24 hr. after injection into the kids (Tables 1 and 4) only about 1·5 % of the dose remained in the body tissue, whereas 2, 5·5 and 8 hr. after injection about 29, 21 and 11 % respectively of the dose remained in the tissues.

Circulating levels of hexoestrol. In Fig. 1 the concentration of circulating hexoestrol in kids 3, 4 and 5 is plotted against time after injection. A figure of 70 ml. of blood/kg. body weight (Courtice, 1943) was used in making these calculations. In the kids the concentration of hexoestrol in the blood reached a maximum 2 hr. after the injection. Owing to steepness of the curves about the maximum and the infrequency of taking blood samples it is possible that the true maxima were considerably higher than indicated. Nevertheless, for kid 5 the maximum corresponds to the circulation of 22 % of the dose. These blood curves also confirm the rapid absorption of the labelled hexoestrol from its site of injection and its nearly complete clearance from the blood within 24 hr. Unfortunately the 0·9 μg. of circulating hexoestrol (3·6 % of dose; see Table 2) in the kid killed 2 hr. after injection does not correspond with the blood hexoestrol levels found for the other kids at that time; thus it is not likely that any animal was killed at the time of the maximum level of circulating hexoestrol. Of the five kids, that in which 2·0 μg. of hexoestrol (8·2 % of the dose; see Table 2) was circulating when it was killed 5·5 hr. after injection had the highest circulating level at the time when samples of body tissues were taken.

In the lambs, from which blood samples were taken more frequently, the increase in concentration of circulating hexoestrol was not as rapid as in the kids (Fig. 1). It was still increasing in the first lamb when killed 4 hr. after injection, and in the second lamb the maximum concentration occurred 3 hr. after injection. Thus there appears to be considerable variation in the rate of absorption of the injected hexoestrol, or in its rate of removal from the blood stream or in the rate of both these processes. In the two lambs which received the divided dose the concentration of hexoestrol in the blood did not reach a steady value as expected but increased up to the time of slaughter, 8 hr. after

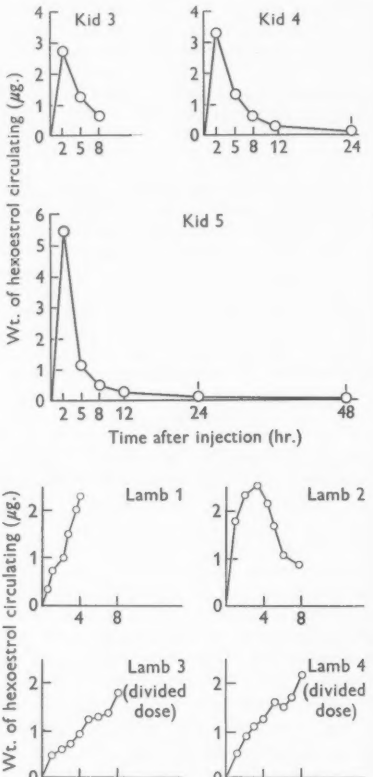


Fig. 1. Circulating amounts of hexoestrol in female kids and lambs at various intervals after its subcutaneous injection; there was assumed to be 70 ml. of blood/kg. body wt. See text and Table 1 for details of treatment of the animals.

the first dose. At this time they had approximately the same amount of the hexoestrol circulating as did the first lamb killed 4 hr. after a single dose.

Concentration of hexoestrol in specific organs and tissues. Table 2 shows the concentration of hexoestrol in the organs and tissues studied. It was impossible to dissect the immature mammary glands free from the fat in which they lay embedded and which accumulated virtually no hexoestrol (Table 2). To obtain a value for the concentration of hexoestrol in the fat-free gland, nitrogen determinations were therefore made on each of the samples and the hexoestrol concentrations corrected to 11 % of nitrogen, which was the average nitrogen content of the dry matter of other tissues studied.

Table 2 shows that, apart from differences attributable to times of killing, the general pattern of distribution of hexoestrol was similar in all animals, both kids and lambs. The concentration in the intestine of lamb 3 is anomalously high, however, especially as the concentrations in other corresponding organs of lambs 3 and 4, on which identical experiments were done, are about the same. After a divided dose (lambs 3 and 4) the concentration in most organs was 1·5–2·5 times as high as that after a single dose (lamb 2).

Although many more animals would have to be used for a detailed study of the changes with time in the concentration of hexoestrol in various organs, the experiment on the kids does give some idea of the results which would then be obtained. In the animal killed 2 hr. after the injection the whole uterus and vagina contained the highest concentration, followed by kidney, liver, intestine and ovaries. In the animal killed 5·5 hr. after injection the kidney had the highest concentration, closely followed by whole uterus and then ovaries and vagina. The high concentration in kidney tissue at this time is probably the result of a high rate of excretion (see below), because in the animal killed 3 hr. later, when excretion had passed its maximum, the concentration of hexoestrol in the kidney was only one-quarter of that of the vagina (which in this animal was the organ with the highest concentration) and occupied sixth place in the descending order of concentration.

Table 2. *Concentration of hexoestrol in various organs and tissues*

See text and Table 1 for details of treatment of the animals.

	Hexoestrol (μmg./g. of dry matter)								
	Kids					Lambs			
Animal no.	1	2	3	4	5	1	2	3	4
Time after injection (hr.)	2	5·5	8	24	48	4	8	8	8
Dose	Single	Single	Single	Single	Single	Single	Single	Divided	Divided
Organ or tissue									
Uterus (endometrium)	14·1	21·1	7·3	1·9	0·3	9·4	7·8	13·8	13·6
Vagina	13·8	12·3	12·0	2·3	0·4	9·5	7·4	10·7	10·8
Uterus (myometrium)	17·7	19·7	6·8	1·4	0·3	5·8	6·0	13·0	12·6
Kidneys	8·6	21·8	3·0	1·2	0·2	10·5	6·2	12·3	17·7
Oviducts	—	—	—	—	—	5·4	4·9	7·0	6·3
Ovaries	4·7	14·1	4·5	1·5	0·4	3·8	4·6	4·8	4·4
Mammary glands*	4·6	9·1	3·7	0·9	0	6·7	3·7	6·5	7·0
Intestine	5·0	7·3	2·2	0·3	0	4·7	3·3	17·5	5·9
Liver	7·0	5·2	2·8	0·6	0·3	6·8	2·9	3·9	5·1
Pituitary gland	3·6	2·6	0·9	0·6	0·1	4·2	1·7	2·8	2·9
Lungs	3·2	3·2	0·8	0·2	0·1	1·8	0·9	1·4	2·5
Lymph nodes	—	—	—	—	—	1·4	0·8	1·4	1·2
Adrenal glands	0·9	1·9	0·4	0·2	0	1·9	0·7	0·9	1·4
Skin	0·8	0·9	0·6	0·4	0·3	0·5	0·4	0·7	0·8
Salivary glands	1·2	1·6	0·5	0·1	0·1	—	—	—	—
Thymus	—	—	—	—	—	1·0	0·4	0·8	0·7
Heart	—	—	—	—	—	1·0	0·4	0·9	1·0
Spleen	—	—	—	—	—	0·6	0·4	0·7	0·6
Pancreas	1·0	0·9	0·4	0·1	0·1	—	—	—	—
Skeletal muscle	2·8	0·5	0·3	0	0	0·5	0·5	0·3	0·4
Brain	—	—	—	—	—	0·3	0·2	0·4	0·4
Bone	—	—	—	—	—	0·2	0·1	0·2	0·2
Fat (perinephric)	0·4	0·1	0·1	0	0	0·2	0·2	0·2	0·2
Blood solids	4·7	8·7	2·2	0·5	0·1	5·6	2·7	5·6	6·5

* Since samples of mammary glands were heavily contaminated with fat, the figures were corrected to 11·0 % nitrogen, the average nitrogen content of the other organs studied.

To compare their capacity for accumulating hexoestrol the mean concentrations in various organs for all the animals except kid 5 have been calculated, as shown in Table 3, the concentration in uterine endometrium being set arbitrarily at 100. Kid 5 was omitted because the hexoestrol concentration in the organs was too low for differences to be significant (see Table 2). The highest concentration is in the organs which respond physiologically to hexoestrol (uterus and vagina) and in the kidneys, which excrete it. In the oviducts, ovaries, mammary glands, intestine and liver the concentration was about half that in endometrium. In the pituitary gland, which is also an organ which responds to oestrogens, the concentration was about one-quarter of that in the uterine endometrium, but was still considerably higher than in many other tissues. The lungs also appeared to have a slight ability to concentrate hexoestrol, whereas in lymph nodes, adrenal glands, skin, salivary glands, thymus and heart the concentration was only 7–10 % of that in uterine endometrium. The concentration in other organs and tissues including spleen, pancreas, skeletal muscle, brain, bone and perinephric fat was only 1–5 % of that in uterine endometrium. The radioactivity of some of these organs may have been due to residual blood solids whose mean specific activity was about 40 % of that of uterine endometrium. It is obvious, however, that there was a true selective accumulation in reproductive organs, since only a small fraction of their radioactivity could have arisen from residual blood. As the concentration in the pituitary glands of the lambs, though relatively low, was on the average nine times that in the portion of the brain to which it was attached, it was probably also due to a selective accumulation.

Table 3. *Relative concentration of hexoestrol in dry matter of various organs and tissues*

Mean concentrations in all tissues assayed (except those of kid 5 see text) were calculated on the basis of 100 for the concentration in uterine endometrium.

Organ	No. of animals	Mean relative concentration (±S.E.)
Uterus (endometrium)	8	100
Vagina	8	99·3±11·4
Uterus (myometrium)	8	88·9±6·7
Kidneys	8	84·9±10·4
Oviducts	4	54·3±3·6
Ovaries	8	50·9±6·3
Mammary glands	8	48·9±3·8
Intestine	8	47·3±11·9
Liver	8	39·9±5·3
Pituitary gland	8	23·7±3·8
Lungs	8	14·8±1·7
Lymph nodes	4	11·0±1·3
Adrenal glands	8	9·7±1·6
Skin	8	7·6±2·0
Salivary glands	4	7·0±0·7
Thymus	4	6·6±1·3
Heart	4	7·4±1·2
Spleen	4	5·2±0·4
Pancreas	4	5·6±0·6
Skeletal muscle	8	5·4±2·2
Brain	4	2·9±0·1
Bone	4	1·6±0·2
Perinephric fat	8	1·5±0·3
Blood solids	8	39·2±3·8

The concentration–time curves for selected organs, and for bile and bladder urine of the kids are shown in Fig. 2. Each point as plotted represents a single animal and the exact shapes of the curves would therefore have to be verified by using more animals at each time interval. The maximum concentration of hexoestrol in muscle and liver was found in the animal killed 2 hr. after the injection. This was true also of pituitary gland, perinephric fat and possibly vagina, although the difference in concentration in vaginal tissue from the first two kids killed (2·0 and 5·5 hr. after injection) was very small (Table 2). The maximum concentration in endometrium and kidney was found in the animal killed 5·5 hr. after injection, as it was also in ovary, mammary gland, intestine and adrenal glands. The remaining organs assayed (lungs, skin, salivary glands and pancreas) had about the same concentration in the two animals first killed, although it was higher than in those killed later. As the concentration in blood solids was not highest in the animal killed 2 hr. after injection, as would be expected from concentration–time curves of kids 3, 4 and 5 (Fig. 1), the significance of the times of these maxima is doubtful. It may well be, however, that the organs showing most true accumulation reach their maximum concentration later than others.

Fig. 2 also shows that most of the radioactivity of the kidney and the liver could have been caused by the residual urine and bile. The specific activity of the bladder-urine solids, which may have been somewhat greater or less than that of the urine actually in the kidney at the time of killing, was in all the kids from 20 to 80 times that of the dry matter of the kidney. Similarly the specific activity of bile solids from the gall bladder was 40–120 times that of the liver dry matter. These graphs show that although the reproductive organs and kidneys consistently showed the greatest accumulation of hexoestrol it was only transient and had disappeared within 24 hr. There was, however, a tendency for the ratio of uterine concentration to blood concentration to increase as the blood concentration itself decreased.

In Table 4 are expressed the estimated percentages of the total dose of hexoestrol which occurred in certain organs and tissues at various times after injection. Some of the values are approximations

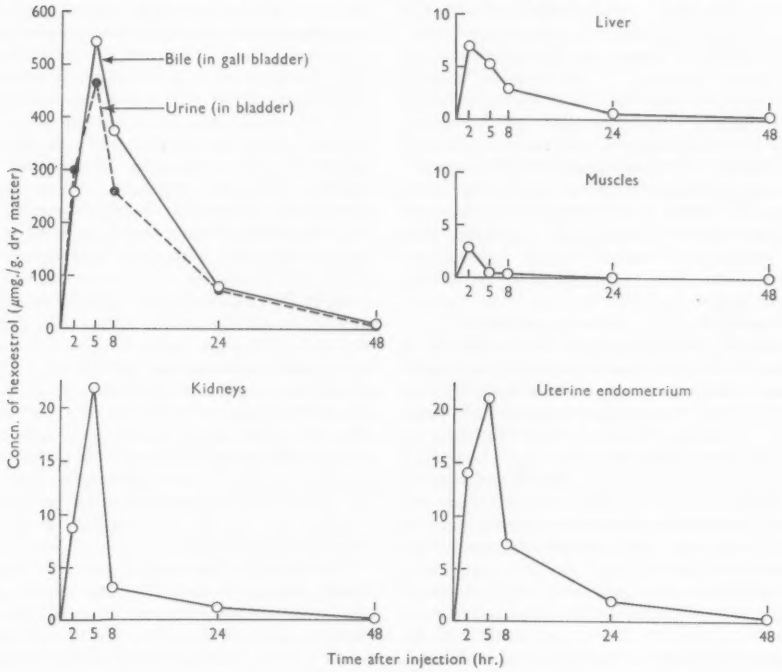


Fig. 2. Hexoestrol concentration–time curves for bile, urine and selected organs from the kids. Each point represents a single animal killed at that time. Each kid received a single subcutaneous injection of 25 μg. of tritium-labelled hexoestrol.

Table 4. *Distribution of hexoestrol in whole organs*

See text and Table 1 for details of treatment of the animals.

	Estimated percentage of total dose in organ								
	Kids					Lambs			
Animal no.	1	2	3	4	5	1	2	3	4
Body wt. (kg.)	14·3	17·7	22·8	14·0	14·2	30·4	25·0	23·9	25·9
Time after injection (hr.)	2	5·5	8	24	48	4	8	8	8
Dose	Single	Single	Single	Single	Single	Single	Single	Divided	Divided
Organ or tissue									
Uterus	0·06	0·17	0·05	0·01	0·001	0·05	0·05	0·09	0·07
Vagina	0·05	0·04	0·04	0·01	0·001	0·03	0·04	0·05	0·05
Kidneys	0·5	1·2	0·25	0·1	0·01	1·2	0·6	1·4	2·1
Liver	2·2	1·9	1·4	0·2	0·08	4·2	1·8	2·6	3·1
Intestine	1·1	2·1	0·8	0·1	—	2·4	1·4	6·9	2·5
Lungs	0·4	0·4	0·2	0·02	0·01	0·4	0·2	0·4	0·5
Heart	—	—	—	—	—	0·2	0·06	0·2	0·2
Skeletal muscle	19·4	4·5	3·4	—	—	7·8	6·4	3·7	5·3
Skin	1·4	2·2	1·9	0·7	0·6	1·7	1·2	1·8	2·1
Bone	—	—	—	—	—	2·5	1·1	1·7	1·8
Blood	3·6	8·2	2·5	0·4	0·07	10·2	3·7	7·5	9·0
Total body tissues	28·7	20·7	10·5	1·5	0·8	30·7	16·6	26·3	26·7

only. For example, considerable portions of uterine and vaginal tissues were retained as uncomminuted connective tissue (see Methods section). Assay of some samples of the washed uncomminuted tissues showed that they had about half the specific activity of the homogenizable portion. This may be of significance and deserves further study in relation to oestrogenic effects on connective tissues (Joseph, Engel & Catchpole, 1954; Asboe-Hansen, 1958). To estimate total concentrations of hexoestrol the assumption was made that 50% of uterine and vaginal tissues were homogenizable and that the dry matter of the remaining tissues had half the specific activity of the homogenizable portion. Only a small portion of other organs was not homogenizable. The methods of calculating the total radioactivities of such organs as intestine, skeletal muscle, skin, bone and blood and their possible inaccuracies have already been discussed. It is felt, however, that the data in Table 4 are reasonably accurate and show that even though the organs such as uterus and vagina, which respond to oestrogens, accumulated the hexoestrol, the maximum amount accumulated in the whole organ was only a small fraction of the total dose (less than 0·2%). At the same time, because of their much greater size, organs such as liver, kidney and intestine accumulated as much as 1–7% of the total dose. There appeared to be an early (2 hr.) accumulation of hexoestrol in skeletal muscle which, however, is unconfirmed, since it was observed only in a single animal. Also a slower turnover of the oestrogen in skin than in most other organs is indicated.

DISCUSSION

That the dose used in this work was physiological is supported by calculations made from body weights and known values for rats (Dodds *et al.* 1944) and cows (F. Glover, personal communication), and also by the work of Robinson, Moore & Binet (1956), who found that the physiological dose of oestradiol benzoate for a ewe was 20 μg. Oestradiol benzoate and hexoestrol have about the same oestrogenic activity when administered subcutaneously (Emmens, 1940–41). Unfortunately, whether or not a physiological response had been produced was not observed in this work. Immature animals were used because it was supposed that, as in the rat (Wiberg & Stephenson, 1957), the uterus of goats and sheep is especially sensitive to oestrogens at this stage of growth.

The results on the excretion of physiological doses of hexoestrol by kids and lambs are in general agreement with those obtained in previous work on rats and rabbits (Dodds *et al.* 1958). The injected hexoestrol was rapidly excreted in both urine and faeces and only a small portion of the dose remained in the body tissues after 24 hr. Also in agreement with the work on rats (Dodds *et al.* 1958), little or none was completely oxidized. The importance of the biliary system as an excretory route for oestrogens has been stressed by many workers, as has the significance of an enterohepatic circulation of oestrogens (Cantarow, Rakoff, Paschkis, Hansen & Walkling, 1943; Sandberg & Slaunwhite, 1957). The observation in the present work that the ratio of total hexoestrol in urine to that in the bile, intestinal contents and faeces (Table 1) increased with time after injection, coupled with the finding of significant amounts in intestinal tissues, is in agreement with the supposition that a hepatic–biliary–enteric circulation of hexoestrol occurs in kids and lambs. It could be argued that such a circulation is of importance in maintaining the level of oestrogen in the blood. However, even at physiological doses the rapid excretion of hexoestrol is evidently the predominant metabolic process. Alternatively it could therefore be argued that the rapid excretion of oestrogens is essential to the efficient regulation of processes which respond to them.

Undoubtedly the most important contribution of this work is the discovery that when small doses of a synthetic oestrogen are administered to young female goats and sheep the highest concentration, either of the oestrogen itself or of a non-volatile derivative, is found in the organs which respond physiologically to it or excrete it. Although long suspected, this, as far as the authors know, is the first time that it has been demonstrated. Also of interest is the accumulation in the pituitary gland, which, though less than that in uterus and vagina, was about nine times that in the immediately adjacent brain. This suggests that oestrogens exert their effect on the pituitary gland direct. The concentration in the mammary glands, though again less than in uterus and vagina, is consistent with the fact that the glands respond to oestrogens (see, for example, Folley, 1956). The reason for accumulation in ovaries is not clear. As the ovaries secrete oestrogen one would not expect them to accumulate it. The accumulation in lungs, though low, is nevertheless greater than that in many other organs, and may be associated with the large proportion of epithelial tissue they contain. Turner (1956) found that the lungs of steers which had been fed with stilboestrol contained more oestrogenic activity than organs such as liver, muscle and kidney. The low concentration in the salivary glands was of interest in view of work on the possible relationship of the sex hormones in the saliva of pregnant women to the sex of the foetus. Rapp & Richardson (1952) obtained evidence that circulating androgens but not oestrogens are

secreted in saliva. Green (1954), however, found that after a dose of 1 g. of stilboestrol the saliva of an old man contained 24·4 μg./100 ml. and that of an old woman 5·8 μg./100 ml. Our work suggests that at physiological doses virtually no hexoestrol passes into the saliva of kids.

At the highest observed concentration the total amount accumulated in the uterus was only 0·2 % of the dose, and in the vagina only 0·05 %. This, however, is not wholly surprising in view of the much smaller doses which are effective when applied locally. The very brief accumulation in these organs does not conflict with observations made by other workers on the changes which occur in the uterus even within 6 hr. of giving oestrogen. Examples are increased water imbibition, electrolyte shifts and increased use of carbohydrate (Szego, 1957); increased uptake of serum albumin (Kalman, 1955); increased incorporation *in vitro* of precursors into proteins and nucleic acids and increased activity of amino acid-activating enzymes (McCorquodale & Mueller, 1958). Our work appears to support the protoplasmic-receptor theory of oestrogen action of Szego (1957).

Similar studies to ours have previously been made on rodents but not on goats or sheep. The failure of other workers to demonstrate a localization of oestrogen in the organs which respond to it is, however, probably due to the use of massive doses, coupled with relatively insensitive methods of detection, rather than to the use of a different species or a different oestrogen. In our work the maximum counting rate in the uterus was about 200 counts/min./mg. dry matter; if the hexoestrol had had only one-thousandth of its actual specific activity (that is about the same as that of the [^{14}C]oestrogens used by previous workers), then the radioactivity accumulated in the various organs could not have been detected. It further appears from the work of Budy (1955) and of Twombly & Schoenewaldt (1951) that if the dose had been increased 1000-fold the pattern of distribution would have been greatly altered and more hexoestrol would have been found in tissues such as bone, adrenal glands, lymph nodes and heart than in those organs most sensitive to it. It seems likely that selective localization on the organs which respond occurs only when small amounts are administered. This suggests that there is a limited amount which can be selectively accumulated. However, such a statement could be verified by comparable experiments with larger doses.

The radioactive compound in the samples has been assumed to be hexoestrol for the purposes of calculation although in fact it has not been identified. Workers who have used massive doses of synthetic oestrogens (1 g. or more to a rabbit; e.g. Smith & Williams, 1948; Dodgson, Garton, Stubbs & Williams, 1948) found that up to 70 % of the dose became conjugated with glucuronic acid, much of which was excreted in the urine. They also found some evidence of sulphate formation. Smith & Williams (1948) considered that there was further metabolism to oestrogenically inactive products. Hanahan *et al.* (1953) obtained evidence of conjugation of stilboestrol at much lower doses (5 μg. to a rat). Many workers have observed both conjugation and conversion into chemically different compounds in the metabolism of the natural oestrogens.

SUMMARY

1. Hexoestrol generally labelled in stable positions with tritium was injected subcutaneously into immature female goats and sheep in physiological doses. The excreta and certain body organs and tissues were assayed for radioactivity at various intervals after injection of the animals.

2. The radioactive hexoestrol was rapidly absorbed from the site of injection and excreted into the urine and into the intestinal contents and faeces by way of the bile. Little or none was completely oxidized to water. Only small amounts (< 2 %) remained in the body tissues 24 hr. after injection. The existence of a hepatic–biliary–enteric circulation of hexoestrol or its derivatives was indicated.

3. There occurred marked selective localization of hexoestrol in the organs known to respond to oestrogens (uterus, vagina, mammary glands and pituitary gland), and in the organs of excretion and re-absorption of hexoestrol (kidney, liver, intestine) and also in ovaries and oviducts. Although these organs contained the highest concentration for at least 24 hr., the accumulation was transient with maximum concentrations occurring from 2 to 5·5 hr. after injection, and dropping rapidly to barely detectable levels 48 hr. after injection. The relatively high concentrations in kidney and liver could be attributed to the very high concentration in urine and bile solids. Lungs appeared to have a slight ability to concentrate the hexoestrol, and the other 12 organs and tissues studied, including adrenal glands, lymph nodes, skin, muscle, bone and perinephric fat, had only from 1 to 10 % of the concentration in the uterus and vagina.

4. Although the uterus and vagina had higher concentrations than any other organs, the maximum percentage of the total dose accumulated by them at any time was less than 0·2.

We wish to thank Dr A. T. Cowie for permitting the use of the post-mortem room and for catheterizing some of the animals; and Dr S. J. Rowland for the determination of nitrogen in tissue samples. The skilled assistance of Mr B. W. E. Peaple and Mr G. Jones in the gas counting is also gratefully acknowledged. The work was done while

one of us (W.G.H.) was on leave from the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin, U.S.A. as a Merck Senior Postdoctoral Fellow of the National Academy of Sciences, National Research Council, Washington D.C.

REFERENCES

Asboe-Hansen, G. (1958). *Physiol. Rev.* **38**, 446.

Budy, A. M. (1955). *Arch. int. Pharmacodyn.* **103**, 435.

Cantarow, A., Rakoff, A. E., Paschkis, K. E., Hansen, L. P. & Walkling, A. A. (1943). *Proc. Soc. exp. Biol., N.Y.*, **52**, 256.

Courtice, F. C. (1943). *J. Physiol.* **102**, 290.

Dodds, E. C., Folley, S. J., Glascock, R. F. & Lawson, W. (1958). *Biochem. J.* **68**, 161.

Dodds, E. C., Golberg, L., Grünfeld, E. I., Lawson, W., Saffer, C. M., jun. & Robinson, R. (1944). *Proc. Roy. Soc.* B, **132**, 83.

Dodgson, K. S., Garton, G. A., Stubbs, A. L. & Williams, R. T. (1948). *Biochem. J.* **42**, 357.

Emmens, C. W. (1940–41). *J. Endocrin.* **2**, 448.

Folley, S. J. (1956). *The Physiology and Biochemistry of Lactation.* Edinburgh and London: Oliver and Boyd.

Glascock, R. F. (1954*a*). *Radioisotope Conference*, vol. 1, p. 262. Ed. Johnston, J. E., Faires, R. A. & Millett, R. J. London: Butterworths Scientific Publications.

Glascock, R. F. (1954*b*). *Isotopic Gas Analysis for Biochemists.* New York: Academic Press Inc.

Glascock, R. F. (1956). *Proc. Int. Conf. Peaceful Uses of Atomic Energy*, **12**, 397. New York: United Nations.

Glascock, R. F. & Hoekstra, W. G. (1958). *Proc. 2nd Int. Conf. Peaceful Uses of Atomic Energy*, (in the Press). New York: United Nations.

Green, S. (1954). *Proc. Soc. exp. Biol., N.Y.*, **86**, 653.

Hanahan, D. J., Daskalakis, E. G., Edwards, T. & Dauben, H. J., jun. (1953). *Endocrinology*, **53**, 163.

Joseph, N. R., Engel, M. B. & Catchpole, H. R. (1954). *Arch. Path.* **58**, 40.

Kalman, S. M. (1955). *J. Pharmacol.* **115**, 442.

McCorquodale, D. J. & Mueller, G. C. (1958). *J. biol. Chem.* **232**, 31.

Rapp, G. W. & Richardson, G. C. (1952). *Science*, **115**, 265.

Robinson, T. J., Moore, N. W. & Binet, F. E. (1956). *J. Endocrin.* **14**, 1.

Sandberg, A. A. & Slaunwhite, W. R., jun. (1957). *J. clin. Invest.* **36**, 1266.

Smith, A. E. W. & Williams, P. C. (1948). *Biochem. J.* **42**, 253.

Szego, C. M. (1957). In *Physiological Triggers*, p. 152. Ed. by Bullock, T. H. Washington, D.C.: American Physiological Society.

Turner, C. W. (1956). *J. Anim. Sci.* **15**, 13.

Twombly, G. H. (1951). *Acta Un. int. Cancr.* **7**, 882.

Twombly, G. H. & Schoenewaldt, E. F. (1951). *Cancer*, **4**, 296.

Wiberg, G. S. & Stephenson, N. R. (1957). *Canad. J. Biochem. Physiol.* **35**, 1107.

A Ribonucleoprotein of Skeletal Muscle and its Relation to the Myofibril

By S. V. PERRY and M. ZYDOWO*

Department of Biochemistry, University of Cambridge

(*Received* 12 *February* 1959)

From the work of a number of investigators there is evidence for the association of nucleic acid with preparations of certain myofibrillar proteins. This property is particularly marked in tropomyosin, which can be obtained from a variety of species and muscle types as a crystallizable complex, nucleotropomyosin, containing up to 20% of nucleic acid (Hamoir, 1951*a*, *b*; Sheng & Tsao, 1954). Smaller amounts of ribonucleic acid have been reported to be present in purified myosin preparations (Mihalyi, Laki & Knoller, 1957; Mihalyi, Brodley & Knoller, 1957). The significance of the ribonucleic acid associated with these proteins has been a matter for speculation, but the recent finding (Perry & Zydowo, 1958, 1959) that a ribonucleoprotein is one of the components of the extra protein (Szent-Györgyi, Mazia & Szent-Györgyi, 1955) suggests that the myofibrillar fraction of the cell may be the origin of the ribonucleic acid. This paper is concerned with the characterization of the ribonucleoprotein extracted from the myofibril and its relation to the distribution of ribonucleic acid in the muscle cell.

* Research Fellow of the Rockefeller Foundation 1957–8. Present address: Department of Biochemistry, Medical Academy, Gdansk, Poland.

METHODS

Preparation of myofibrils and muscle granules. Myofibrils were prepared from the back and leg muscles of the rabbit and the breast muscles of the hen by the method of Perry & Grey (1956), modified as described by Perry & Zydowo (1959). Washed granular preparations were prepared from the same sources by the method of Perry & Zydowo (1959).

Preparation of ribonucleoprotein. The dilute extra-protein fraction, prepared as described previously (Perry & Zydowo, 1959), in 0·04 M-KCl containing 6·7 mM-potassium phosphate buffer, pH 7·2, was adjusted to 0·1 M-KCl by the addition of solid KCl and to pH 7·6 with M-2-amino-2-hydroxymethylpropane-1:3-diol (tris). This solution was run into a column of diethylaminoethylcellulose equilibrated with 0·1 M-KCl containing 0·02 M-tris chloride

buffer, pH 7·6, and the ribonucleoprotein eluted between 0·4 and 1·5 M-KCl was collected. This is fraction IV of Perry & Zydowo (1959). The eluted ribonucleoprotein was exhaustively dialysed against distilled water, freeze-dried and stored at −15°. All preparations and chromatographic separations were carried out at 1° to 2°.

Fractionation of muscle. A weighed sample of minced fresh muscle was homogenized for 40–60 sec. in a small homogenizer of the Waring Blendor type with a known amount of medium (usually about 10 vol. of 0·25 M-sucrose or 0·1 M-KCl–borate buffer, pH 7·1). From weighed portions of the homogenate, each containing about 2 g. of muscle, the following fractions were isolated.

(1) Nuclear-myofibrillar. This was the residue sedimenting after 15 min. at 800 *g*, from which soluble proteins (sarcoplasm) and granular material were removed by suspending the residue from the first centrifuging in fresh medium and washing by centrifuging three times. (2) Granular. This fraction was sedimented by centrifuging for 2 hr. at 15 000 *g* the combined supernatant and washings separated from the nuclear-myofibrillar fraction. It was washed once by centrifuging again. (3) Sarcoplasmic. This fraction consisted of the combined supernatant and washings after removal of the granular fraction. It contained small amounts of granular material which could be sedimented by prolonged centrifuging at higher speeds. Fractionations were usually carried through in triplicate.

Chromatography. Diethylaminoethylcellulose was employed as previously described (Perry & Zydowo, 1959). The sample of ECTEOLA–cellulose used (0·25 m-equiv. of N/g.) was prepared by Dr J. P. Waller in this Laboratory by the method of Peterson & Sober (1956). Samples for fractionation on ECTEOLA–cellulose were equilibrated by dialysis against 0·01 M-potassium phosphate buffer, pH 7·05, and applied to a column of the cellulose prepared and equilibrated against the same buffer, as described for diethylaminoethylcellulose (Perry & Zydowo, 1959). The column was developed by applying a gradient of KCl dissolved in 0·01 M-potassium phosphate buffer, pH 7·05. Chromatography was carried out at 1–2°. The chloride concentration of the eluate was estimated by titration with 0·02 N-$AgNO_3$ (Perry & Zydowo, 1959). Application of KCl up to molar concentration eluted about 87% of the total material absorbing at 260 mμ that was applied. M-KOH was required to elute the remaining material from the column.

Estimation of bases in ribonucleoprotein. Separation of bases was achieved by hydrolysis of the nucleoprotein in 72% (w/w) perchloric acid, followed by chromatography on Whatman no. 1 paper as described by Wyatt (1951) with the modification that the hydrolysis was carried out for 2 hr. as recommended by Markham (1955). The amounts of the bases eluted from the paper were estimated spectrophotometrically.

Estimation of nucleic acids. Whole muscle, rapidly removed from the animal and chilled in ice, was prepared for analysis by dissecting it free from obvious connective tissue. A portion (0·5–1·0 g.) of muscle was homogenized in the MSE Masticator (Measuring and Scientific Equipment Ltd., London) with 20 vol. of absolute ethanol, the homogenate was transferred quantitatively to a centrifuge tube and usually washed twice with ethanol and two or three times with ether. Whole homogenates, prepared at 0° with a small homogenizer of the Waring Blendor type, myofibrils and tissue fractions were precipitated with at least 4 vol. of ethanol and subsequently washed by centrifuging twice with ethanol and twice with ether. In some instances the homogenates of whole muscle and the fractions obtained from them were precipitated at 0° with an equal volume of 15% (w/v) trichloroacetic acid before washing with ethanol. The air-dried residues were extracted three times at 0° with about 50 vol. of 2% (w/v) perchloric acid to remove nucleotides. These extracts were discarded and nucleic acids subsequently extracted four or five times with 30–50 vol. of 10% (w/v) perchloric acid at 70°. The completeness of the extraction of both nucleotides and nucleic acid was ensured by measurement of absorption of the extracts at both 260 and 280 mμ.

Total P analysis was carried out by digesting up to 2 ml. of the 10% perchloric acid extracts with 0·5 ml. of 10 N-H_2SO_4. When the contents of the tubes had been concentrated to small volume by an initial heating, digestion was continued for 30 min. on an electric furnace to drive off the perchloric acid, and P estimated under the Fiske & Subbarow (1925) conditions.

Ribose was estimated on the 10% perchloric acid extracts by the orcinol method as modified by Ceriotti (1955). Ribose values were converted into ribonucleic acid P values with a factor determined by analysis of the 10% perchloric acid extract of a pooled sample of the ribonucleoprotein isolated from myofibril preparations (see Results section).

The extinctions at 260 and 268 mμ of the 10% perchloric acid extracts were also measured but these values did not agree well with the amounts of ribose and P present. This was probably due to the extraction of some protein by the 10% perchloric acid. Occasionally with the orcinol reaction a brown colour developed instead of the usual green characteristic for ribose. This was especially noted during analyses of whole dorsal muscle, and of sarcoplasmic and granular fractions from this muscle. Interference was never observed with the myofibrillar fraction. It is possible that this interference was due to other carbohydrates, probably glycogen (Brown, 1946).

The Dische (1930) reaction for deoxyribonucleic acids was found to be too insensitive for most of the 10% perchloric acid extracts.

Other analytical methods. Tropomyosin was assayed by measuring the drop of viscosity under the influence of KCl as described by Perry (1953) and Perry & Corsi (1958), and adenosine triphosphatase activity was measured by the standard procedure used in this Laboratory (Perry & Grey, 1956).

Total N was estimated by the usual micro-Kjeldahl procedure with selenium oxide–$CuSO_4$ mixture as catalyst.

Absorption spectra in the ultraviolet range were obtained with the Beckman Ratio Recording Spectrophotometer.

Materials. All the chemicals used for analytical purposes were of A.R. grade. Orcinol was recrystallized several times from benzene after shaking with charcoal. *iso*Amyl alcohol was redistilled before use. Tris was recrystallized twice from ethanol. Yeast sodium ribonucleate (British Drug Houses Ltd.) was purified according to the method of Woodward (1944).

RESULTS

Composition of ribonucleoprotein fraction

As isolated from the extra-protein fraction of rabbit myofibrils by chromatography on diethylaminoethylcellulose, the ribonucleoprotein (frac-

tion IV, Perry & Zydowo, 1959) was soluble in water and when freeze-dried from salt-free solutions it was readily redissolved at pH 7·0.

Owing to difficulties in isolating sufficient quantities it was not possible, however, to establish precisely by rigorous tests the degree of homogeneity of the ribonucleoprotein fraction. By gradient-chromatography of the extra protein on diethylaminoethylcellulose the nucleoprotein was consistently eluted, at pH 7·6 in 0·02 M-tris chloride buffer, as a sharp, fairly symmetrical peak at a chloride concentration of about 0·7 equiv./l. (Fig. 1). Analysis of the fraction as eluted from diethylaminoethylcellulose between 0·4 and 1·5 M-KCl, the standard procedure used for preparation of the ribonucleoprotein, gave N/P ratios ranging from 3·8 to 4·4. In the ultracentrifuge a sample obtained by combining the ribonucleoprotein isolated from three different preparations of extra protein gave a relatively slowly moving peak ($S_{20,w} \sim 2{\cdot}6$), from which a small faster component had partly emerged after 2 hr. at 59 000 rev./min. in 0·1 M-KCl–0·05 M-potassium phosphate buffer, pH 7·1.

The presence of protein in fraction IV was evident from the biuret test and the fact that the minimum of the ultraviolet-absorption curve (Fig. 2) was in the region of 240 mμ rather than at 230 mμ, the minimum characteristic of pure ribonucleic acid (RNA) (Beaven, Holiday & Johnson, 1955). There was some suggestion of partial fractionation of the ribonucleoprotein by diethylaminoethylcellulose for the wavelength at the point of minimum absorption in the ultraviolet light decreased in samples taken in sequence as the ribonucleoprotein was eluted from the column by progressively increasing the KCl concentration from 0·4 to 1·5 M. Minimum values approaching 230 mμ were reached at the peak of the elution chromatogram, but samples at all stages of the elution contained nucleic acid, for in every case the ratio E_{260}/E_{280} was appreciably greater than one.

Much better separation of protein and RNA was obtained when the ribonucleoprotein was rechromatographed on ECTEOLA–cellulose (Peterson & Sober, 1956). Fraction IV dissolved in 10 mM-potassium phosphate buffer, pH 7·05, was held on ECTEOLA–cellulose and subsequent gradient-elution with KCl gave two peaks (Fig. 3). The first and smaller peak, as judged by absorption at 280 and 260 mμ, was eluted at a chloride concentration of 0·1 equiv./l., and the second at 0·3 equiv./l. For the first peak the ratio E_{260}/E_{280} barely exceeded 1, which from the data obtained by Warburg & Christian (1941–42) with enolase would indicate that less than about 5 % of nucleic acid was present.

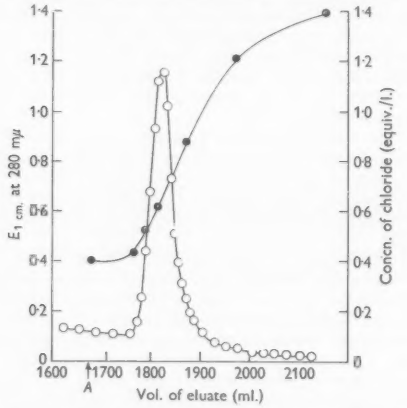


Fig. 1. Gradient-elution of the nucleoprotein from rabbit myofibrils on diethylaminoethylcellulose. Extra protein (72·5 ml.; $E_{1\,cm.}$ 13·2 at 280 mμ) was concentrated by precipitation with saturated $(NH_4)_2SO_4$, and was dissolved in 0·1 M-KCl–0·02 M-tris chloride buffer, pH 7·6, and applied to a column 27 cm. × 2 cm. diam. The bulk of the protein was previously removed by elution with 0·4 M-KCl–0·02 M-tris buffer, and a gradient to 2·0 M-KCl–0·02 M-tris buffer was applied at the point *A*. ○, $E_{280\,m\mu}$; ●, chloride concentration.

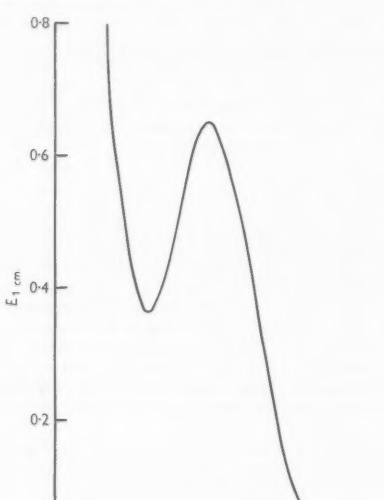


Fig. 2. Ultraviolet-absorption spectrum of the nucleoprotein isolated from rabbit myofibrils; in 1·5 M-KCl–0·02 M-tris chloride buffer, pH 7·6.

The peak eluted at higher salt concentration had a ratio E_{260}/E_{280} about 2 and was relatively pure RNA, although the minimum of the absorption curve was not exactly at 230 mμ, which suggested that small amounts of protein were still present.

Ribonucleic acid component

Earlier investigations (Perry & Zydowo, 1959) indicated that there was no significant amount of deoxyribonucleic acid in the ribonucleoprotein fraction. Quantitative chromatographic separation of the bases liberated by hydrolysis in 72% (w/w) perchloric acid (Wyatt, 1951) showed the composition of ribonucleic acid obtained from myofibrils isolated from the mixed back and leg muscles of the rabbit to be relatively constant. These results are summarized in Table 1, in which is also included the base analysis of the corresponding ribonucleoprotein isolated from myofibrils prepared from hen-breast muscle. The composition of the RNA is remarkably similar in both species and is characterized by a high-guanine and low-uracil content, features which are characteristic of RNA from most animal species.

Yeast nucleic acid which has been partly degraded by ribonuclease is, however, also characterized by a relatively high guanine content (Magasanik & Chargaff, 1951; Markham & Smith, 1952; Volkin & Cohn, 1953), and it was suggested by Dr R. Markham that the high content of this purine in the myofibrillar RNA might be indicative of partial degradation of the muscle nucleic acid by action of intracellular enzymes during preparation. It seemed very unlikely that much degradation of the myofibrillar RNA had occurred, for all preparations were made at 0° and the ribonuclease activity of skeletal muscle is known to be very low (Bain & Rusch, 1944; Greenstein & Thompson, 1943; Roth & Milstein, 1952). As a control experiment a solution of yeast RNA, previously purified to remove degraded material, was incubated for 2 hr. at 25° with a rabbit skeletal-muscle homogenate made in 0·1 M-KCl containing 39 mM-borate buffer, pH 7·1 (Perry, 1953). No significant increase in acid-soluble material absorbing at 260 mμ could be detected after this incubation, and the ratio of the bases present in the acid-insoluble yeast RNA was unchanged.

Excellent agreement was obtained between the amount of ribose estimated by the orcinol method per μg.atom of total phosphorus in the ribonucleoprotein and that predicted from the base analysis. On the basis that only ribose which is purine-linked is estimated under the conditions of the orcinol reaction, the analyses in Table 1 indicate that for every μg.atom of rabbit RNA phosphorus, 0·532 μmole of ribose should be estimated by the orcinol reaction. With the sample used as the standard in these studies 0·531 μmole of ribose was in fact estimated/μg.atom of total RNA phosphorus. Analysis of another sample of the ribonucleoprotein gave a value of 0·527 μmole of ribose/μg.atom of total RNA phosphorus.

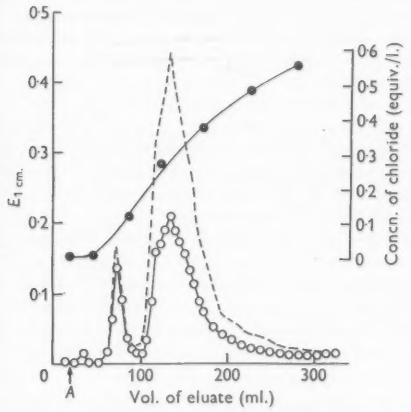


Fig. 3. Chromatography of ribonucleoprotein on ECTEOLA–cellulose. A sample (2 ml.) of ribonucleoprotein solution ($E_{1\,cm.}$ 17·62 at 260 mμ) in 0·01 M-potassium phosphate buffer, pH 7·05, was applied to 1 g. of the cellulose in a column (1 cm. diam.). Gradient to 0·7 M-KCl–0·01 M-phosphate was applied at the point *A*. ○, $E_{280\,m\mu}$; - - -, $E_{260\,m\mu}$; ●, chloride concentration.

Protein component

With the values for total nitrogen (16·74) and phosphorus (9·57) contents of the myofibrillar RNA calculated from the base analyses (Table 1), figures can be obtained for the non-RNA nitrogen in the ribonucleprotein from the data on elementary analyses quoted earlier. Assuming that all the phosphorus present is derived from RNA, the nucleic acid accounts for 41–47% of the total nitrogen. The additional nitrogen was presumed to be due to protein, as it was non-dialysable, the

Table 1. *Base composition of ribonucleoprotein isolated from the extra-protein fraction of skeletal myofibrils*

Results are expressed in moles/100 moles of total bases. Results for the rabbit are averaged from seven estimations, made on three different samples; those for the hen are from four estimations on the same sample. Results are given ±S.E.M.

	Rabbit	Hen
Guanine	35·6±0·59	35·1±0·75
Adenine	17·6±0·34	19·9±0·21
Cytosine	28·6±0·55	28·8±0·78
Uracil	18·2±0·61	16·2±0·10

material was biuret-positive and possessed the general properties of protein. In one experiment 32 % of the total N was not extracted by hot 10 % perchloric acid, suggesting that at least this proportion of the nitrogen was due to protein. The fractionation on ECTEOLA–cellulose indicated a protein/RNA ratio comparable with that obtained from the nitrogen and phosphorus analyses, in so far as it was estimated that 50 % of the total material eluted was in the peak eluted at the lower ionic strength.

The nature of the protein component was not established but the chromatographic behaviour of the bulk of the sarcoplasmic components, myosin, actin and tropomyosin (Perry & Zydowo, 1959; Perry, 1959, unpublished work), all of which were eluted from diethylaminoethylcellulose at pH 7·6 by KCl at concentrations below 0·4 M, suggested that it was not identical with any of these. In view of the fact that both tropomyosin and myosin preparations have been reported to contain RNA the possibility of the identity of the protein moiety with either of these myofibrillar proteins was further investigated. After equilibration by dialysis against 78 mM-borate buffer, pH 7·1 (Perry & Corsi, 1958), the solution of a pooled sample of ribonucleoprotein was slightly viscous, relative viscosity at $0° = 1·1$ ($E_{1\,cm.}$ 25·33 at 260 mμ). A sample of yeast RNA of similar extinction, which had been purified to remove depolymerized material by the method of Woodward (1944), had a relative viscosity of 1·01. Addition of KCl to 0·5 M reduced the relative viscosity of the ribonucleoprotein to 1·05, whereas the viscosity of yeast nucleic acid was virtually unchanged. A fall in viscosity on increasing the ionic strength is characteristic of tropomyosin. Nevertheless, the viscosity effects obtained with the ribonucleoprotein were very small and certainly not large enough to be used for reliable assays of tropomyosin, but if the whole fall in viscosity was due to this protein the amount present did not account for more than 10–15 % of the non-nucleic acid nitrogen. It can be concluded that tropomyosin, if indeed present at all in the ribonucleoprotein fraction, is only a minor component.

The absence of any adenosine triphosphatase activity and the solubility characteristics of the ribonucleoprotein strongly suggested, in addition to the facts given above, that myosin was not the protein associated with the nucleic acid.

Origin of the ribonucleoprotein

The ribonucleoprotein was found to be present in the extra-protein fraction of myofibrils isolated from rabbit skeletal muscle and from breast muscle of the hen, the only other species so far studied. This fact, coupled with the results of RNA determinations on isolated myofibrils (see Table 2), indicated that the ribonucleoprotein was a consistent and significant component of all myofibril preparations.

On extraction of myofibrils with the pyrophosphate–KCl solution (Hasselbach & Schneider, 1951) used in the preparation of the extra protein, about 60 % of the RNA, and protein consisting mainly of myosin and representing in all about 70 % of that present in the myofibril, passed into solution. As most of the RNA, however, stayed in solution when the (acto)myosin was subsequently precipitated from this extract it seemed that under these conditions at least there was not a particularly strong affinity between the two proteins. On the other hand, the bulk of the actin and tropomyosin could be extracted from myofibrils by treatment with 5 mM-tris chloride buffer, pH 8·1 (Perry & Corsi, 1958), accompanied by only about 15 % of the myofibrillar RNA.

Formed elements in the cell which are known to contain ribonucleoprotein and which would conceivably contaminate the myofibrillar fraction are nuclei and the endoplasmic reticulum. Although nuclei are seldom seen in myofibril preparations the latter contained 630–970 μg. of deoxyribonucleic acid phosphorus/g. of total nitrogen, indicating that appreciable contamination was present. The ratio of RNA to deoxyribonucleic acid (DNA) in nuclei does not, however, exceed 1:4 (Dounce, 1955), and it can be concluded from the RNA and deoxyribonucleic acid concentrations in myofibrils that probably not more than about 10 % of the total RNA is of nuclear origin.

Rabbit skeletal muscle contains few granules comparable in size with the mitochondria of liver and of the more oxidative muscles. Under conditions adequate to sediment the mitochondria in these tissues extremely low yields of granules are obtained from rabbit-muscle homogenates, and it is

Table 2. *Ribonucleic acid and total nucleic acid content of whole muscle and myofibrils from the rabbit*

S.E.M. values are given when analysis has been carried out on more than two different animals or preparations. Figures in parentheses indicate the number of determinations.

	Ribose	RNA phosphorus	Total nucleic acid phosphorus
Whole psoas (μg./g. wet wt.)	262±27·8 (4)	102±10·8 (4)	123±10·9 (4)
Whole latissimus dorsi (μg./g. wet wt.)	210 (2)	81 (2)	113±14·4 (5)
Myofibril preparation (μg./mg. of total N)	4·37±0·59 (3)	1·70±0·23 (3)	2·81 (2)

difficult to decide upon the appropriate conditions to sediment the mitochondrial fraction to separate it from the fraction corresponding to the microsome fraction of other tissues.

Although few granules were apparent in washed myofibril preparations, the possibility was considered that the myofibrillar ribonucleoprotein could arise from contamination with the small granules which can be seen on microscopic examination of sarcoplasmic extracts. The granules prepared from a muscle homogenate in 0·1 M-KCl–39 mM-borate buffer, pH 7·1, by centrifuging for 2 hr. at 15 000 *g* were extracted in the same way as for the preparation of the extra protein, and the resulting solution was chromatographed on diethylaminoethylcellulose in the manner used to fractionate the extra protein (Perry & Zydowo, 1959). Fig. 4 illustrates the result of such an experiment and shows the relatively small amount of ribonucleoprotein which was extracted from these granules compared with that from myofibrils treated in the same way (Perry & Zydowo, 1959). It was concluded that even if all of the granules sedimenting after 2 hr. at 15 000 *g*, which were originally present in the muscle, remained with the myofibrils during their preparation, the granules would account for only about one-fifth or one-sixth of the total ribonucleic acid usually found in the extra-protein fraction. This clearly excluded contamination with the larger granules as a source of the myofibrillar ribonuclear protein, as do also the fractionation studies described below.

Information about the endoplasmic reticulum has been obtained mainly with liver cells and chemical data about its presumed equivalent in muscle cells, the sarcoplasmic reticulum, is lacking. In most cells the bulk of the cytoplasmic ribonucleoprotein is associated with the endoplasmic reticulum fraction, which requires prolonged centrifuging at high speeds for sedimentation (Loftfield, 1957). High-speed centrifuging of sarcoplasmic extracts from rabbit skeletal muscle did not yield a microsome fraction containing the bulk of the RNA of the cell (see below), but most of the RNA sedimented at low speeds with the myofibrillar-nuclear fraction. The myofibrillar ribonucleoprotein did not arise from contamination with the sarcoplasmic proteins which are still present in well-washed myofibrils, for little RNA could be demonstrated in the sarcoplasmic fraction either by analysis or chromatography. When the fraction of the cytoplasm not sedimented after 2 hr. at 15 000 *g* (consisting of the soluble proteins and probably some sarcoplasmic reticulum) is fractionated on diethylaminoethylcellulose no significant amounts of ribonucleoprotein are eluted at higher KCl concentrations (Perry & Zydowo, 1959).

Distribution of nucleic acid within the muscle cell

Although the values in the literature for the nucleic acid content of muscle vary considerably from author to author, in all cases the amounts present are relatively low compared with those present in other tissues. An earlier study (Perry, 1952) indicated that significant amounts of RNA phosphorus were present in skeletal myofibril preparations, and Needham & Cawkwell (1957) have found RNA in the fraction of rat uterus that is

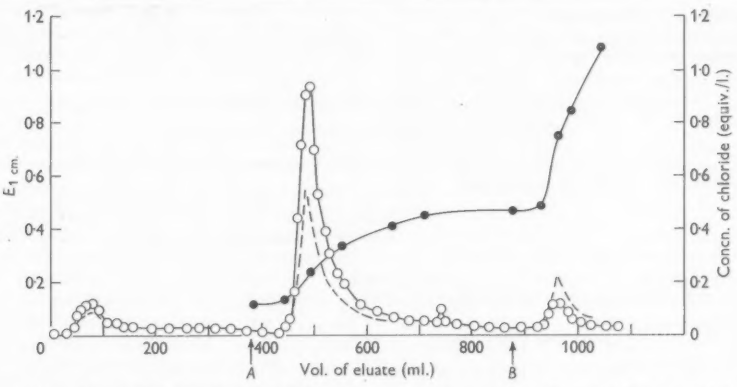


Fig. 4. Chromatography on diethylaminoethylcellulose of extract of granules sedimented from a rabbit sarcoplasmic extract by centrifuging for 2 hr. at 15 000 *g*. The extract was prepared in the same way as the extra-protein fraction from myofibrils (see Methods). A sample (50 ml.) of protein solution ($E_{1\,cm.}$ 2·85 at 280 mμ) dissolved in 0·1 M-KCl–0·02 M-tris chloride buffer, pH 7·6, was applied to a column (17 cm. × 2 cm. diam.). Gradients to 0·45 M-KCl–0·02 M-tris and to 1·5 M-KCl–0·02 M-tris buffer were applied at points *A* and *B* respectively. ○, $E_{280\,m\mu}$; - - -, $E_{260\,m\mu}$; ●, chloride concentration.

insoluble at lower ionic strengths, which presumably contains the contractile proteins of this tissue. More detailed investigation of the ribonucleic acid content of whole muscle and myofibril preparation indicated that even after repeated washings and centrifugings the myofibrils contained ribonucleic acid, which, if it was all associated with these structures *in situ*, represented almost half that present in the whole muscle (Table 2). Satisfactory RNA ribose estimations were obtained with rabbit psoas muscle, but estimations on whole longissimus dorsi muscle and the granular and sarcoplasmic fractions isolated from it frequently suffered from interference owing to a brown which developed under the condition of the orcinol reaction for ribose. In consequence only very few satisfactory values for the RNA ribose content of longissimus dorsi muscle were obtained. Nevertheless these were comparable with the values obtained for the psoas. Considerable variation in values was noted between animals, as has also been reported for the rat by Schmidt & Schlieff (1956).

To discover more precisely how the nucleic acid was distributed within the muscle cell, longissimus dorsi muscle, which formed the bulk of the tissue used for myofibril preparations, was homogenized both in 0·25 M-sucrose and in 0·1 M-KCl–39 mM-borate buffer, pH 7·1, and separated into myofibrillar-nuclear and so-called granular and sarcoplasmic fractions (see Methods). Table 3 indicates the distribution of total nitrogen between these fractions and indicates the relatively small amount of nitrogen sedimenting after 2 hr. at 15 000 *g*, conditions far in excess of those required to sediment mitochondria in other tissues. It is difficult to generalize about muscle from experience with other tissues, but some microsomes might be expected to sediment in this granular fraction. In one experiment, on further sedimentation of the sarcoplasmic fraction obtained by homogenization in 0·1 M-KCl–39 mM borate buffer, pH 7·1, an amount of material representing 1·7 mg. of nitrogen/g. wet wt. of muscle was obtained after 1 hr. at 100 000 *g*, conditions which would be expected to sediment the microsome fraction. It is worth noting that the sarcoplasmic fraction was a smaller proportion of the total nitrogen when fractionation was done in 0·25 M-sucrose rather than in the ionic medium.

The total phosphorus determinations made on hot 10 % perchloric acid extracts of the fractions clearly indicate that about 70 % of the total nucleic

Table 3. *Distribution of total nitrogen between fractions of homogenates of rabbit longissimus dorsi muscle*

S.E.M. values are given when analysis has been carried out on more than two samples from different animals. Figures in parentheses indicate the number of determinations.

	Myofibrils, nucleus	Granules	Sarcoplasm
Homogenized in 0·1 M-KCl–39 mM-borate buffer			
Total nitrogen (mg./g. wet wt.)	20·6±0·42 (5)	1·7±0·15 (4)	15·2±0·14 (4)
Percentage of total nitrogen	55	5	40
Homogenized in 0·25 M-sucrose			
Total nitrogen (mg./g. wet wt.)	23·9 (2)	1·3 (1)	11·0 (1)
Percentage of total nitrogen	66	4	30

Table 4. *Distribution of nucleic acid between fractions obtained from homogenates of rabbit longissimus dorsi muscle*

Results are expressed in μg./g. wet wt. of muscle and, except for the figures in parentheses, are average values obtained from fractionations each carried out in triplicate.

		(1) Myofibrils nucleus	(2) Granules	(3) Sarcoplasm	(4) Whole muscle	(5) Recovery (1), (2) & (3)
Homogenized in 0·25 M-sucrose						
Expt. 1	RNA phosphorus	65·3	4·2	—	—	—
	Total P of 10 % perchloric acid extract	68·1	9·5	9·7	—	87·3
Expt. 2	RNA phosphorus	57·7	6·1	—	82·6	—
	Total P of 10 % perchloric acid extract	82·5	15·5	19·4	123·8	117·4
Total P, distribution (%)		74	12	14	—	—
Homogenized in 0·1 M-KCl–39 mM-borate buffer						
Expt. 3	RNA phosphorus	49·0	(4·9)	13·8	81·0	77·7
	Total P of 10 % perchloric acid extract	59·7	9·9	11·0	86·8	80·6
Expt. 4	RNA phosphorus	55·6	—	(16·5)	—	—
	Total P of 10 % perchloric acid extract	63·1	23·7	14·5	96·1	101·3
Total P, distribution (%)		68	18	14	—	—

acid was carried down with the myofibrillar fraction irrespective of whether sucrose or an ionic medium was used for fractionation (Table 4). From the limited ribose determinations obtained it appears that about 60 % of the ribonucleic acid is likewise associated with the myofibrillar-nuclear fraction. These results apply in both ionic or non-ionic media; in fact rather more nucleic acid is sedimented at low speeds when the homogenate is made in 0·25 M-sucrose than when it is made in the KCl–borate buffer medium.

DISCUSSION

In the rabbit skeletal-muscle cell a large part of the total RNA is associated with the myofibrillar-nuclear fraction obtained on low-speed centrifuging of homogenates of this tissue. As most of this RNA is still present in myofibrils which have been subjected to further purification and repeated washing by centrifuging, it appears that there is a fairly intimate association between the nucleic acid and these structures. The bulk of the myofibrillar RNA exists as a ribonucleoprotein which is present in the extra-protein fraction, and it seems likely that this ribonucleoprotein is the source of the nucleic acid which is present in preparations of myosin and nucleotropomyosin. The base composition of the ribonucleoprotein isolated from the extra protein is very similar to that of the nucleic acid bound to myosin, which has been studied by Mihalyi, Brodley & Knoller (1957). It is difficult to compare many of the properties of the two preparations, as in the latter work the ribonucleoprotein fraction displayed considerable heterogeneity, due no doubt to the fact that it was prepared by somewhat drastic techniques designed to denature preferentially the myosin. Mihalyi, Brodley & Knoller (1957) suggest that a heat-stable protein present in their preparation bears some resemblance to tropomyosin.

The base analyses of the ribonucleic acid component from nucleotropomyosin of various fish muscles (Hamoir, 1951*b*; Tarr, 1958) are also similar to those of the rabbit and hen myofibrillar nucleoprotein. It is likely that nucleotropomyosin is a complex of tropomyosin with ribonucleoprotein rather than with ribonucleic acid alone.

From the limited studies carried out, the protein moiety of the isolated nucleoprotein does not appear to be identical with myosin or tropomyosin, and in view of the ease with which the ribonucleoprotein can be separated by chromatography from myosin (S. V. Perry, unpublished work) and tropomyosin (Perry & Zydowo, 1959), its association with them need not indicate any special significance other than that under conditions for their extraction, the ribonucleoprotein is also extracted and shows some non-specific affinity for both these proteins.

It is difficult to determine the precise localization of the ribonucleoprotein in the muscle cytoplasm. The results exclude nuclear contamination from contributing more than a small fraction of the total myofibrillar RNA, but do not allow us to decide whether the RNA is built into the filamentous structure of the myofibril or is preferentially localized in some other cytoplasmic element such as the sarcoplasmic reticulum which may be attached to the myofibrils or sedimented with them. A number of workers have produced cytochemical evidence for the localization of nucleic acid in the myofibrils. Clavert, Mandel, Mandel & Jacob (1949) claimed to show a specific histochemical-staining reaction for RNA in the A band of myofibrils, whereas Caspersson & Thorell (1942) observed in the I band a concentration of material absorbing at 259 mμ which could be due either to nucleotides or to nucleic acid. Further, Gerendas & Matoltsy (1948) isolated from muscle nucleoprotein-like material which they considered played a part in producing the characteristic birefringent pattern of the myofibril. None of these studies distinguishes between ribonucleoprotein material actually built into the myofibril structure rather than material in other fine structures which may be overlying it.

The studies carried out on the RNA distribution indicate that in muscle homogenates in 0·1 M-potassium chloride–39 mM-borate buffer, pH 7·1, or in 0·25 M-sucrose, the bulk of the cytoplasmic RNA does not sediment in a manner comparable with that of the microsome fraction of liver. On fractionation of muscle the myofibrillar-nuclear fraction contained about 60 % of the RNA of the whole muscle. Some of this nucleic acid may have been lost from myofibrils during the repeated washing and centrifuging employed to purify preparations, for the average RNA content of such preparations is somewhat lower than that of the myofibrillar-nuclear fraction. If this lower figure is taken, and assuming that 1 g. of fresh muscle contains 20 mg. of myofibrillar nitrogen, about 40 % of the total RNA in the cell would be associated with the myofibrils.

Electron-microscope studies have shown reticular material to be abundant and associated with the myofibril in a characteristic fashion in certain types of muscle (Porter & Palade, 1957). This is a marked feature of *Amblystoma* skeletal muscle, but in rabbit skeletal muscle sarcoplasmic reticulum is relatively scarce, judging from the electron-microscope studies in the literature, and the structures which are characteristic of *Amblystoma* reticulum have not been reported. If there is an association of ribonucleoprotein-rich reticulum

with the myofibril it would be expected to be sedimented at low speeds with the myofibrillar-nuclear fraction. Thus in the ease with which ribonucleoprotein-rich material can be sedimented from homogenates muscle resembles hen oviduct tissue (Hendler, 1956) but differs from most animal tissues.

The relatively low ribonucleic acid content of muscle agrees with the observation (K. R. Porter, personal communication) that small granules comparable with the ribonucleoprotein-rich particles associated with the endoplasmic reticulum of other tissues are much less abundant in muscle.

In the ratio of protein to nucleic acid at least the myofibrillar ribonucleoprotein resembles the microsomal ribonucleoprotein particles obtained from other tissues, although its sedimentation behaviour suggests that it is considerably smaller than the microsomal particles of characteristic sedimentation velocity which have been reported in the literature (see Tissières & Watson, 1958, for review). It is not unreasonable to expect the muscle cell, with its special problem of synthesis of the myofibrillar proteins, to have an intimate association between ribonucleoprotein and the myofibrils. The ribonucleoprotein of the myofibrils may fulfil a role in the synthesis of the contractile proteins comparable with that accredited to the ribonucleoprotein of the endoplasmic reticulum of other cells, and it is hoped to explore these possibilities in future work.

SUMMARY

1. A ribonucleoprotein has been isolated as a minor component of skeletal-muscle myofibrils and characterized.

2. Ribonucleic acid of the high-guanine, low-uracil type constitutes about 50% of the nucleoprotein.

3. The bulk of the protein component does not appear to be identical with the known myofibrillar proteins.

4. In muscle homogenates about 60% of the total ribonucleic acid in the cell sediments at low speeds of centrifuging and most of this nucleic acid is associated in some way with the myofibrils.

We wish to express our thanks to Miss Freda Johnson for her skilled technical assistance, to Mr B. Boon for his help in the ultracentrifuge experiments and to Dr J. P. Waller for a gift of ECTEOLA–cellulose. We are also grateful to the Medical Research Council for a research expense grant (to S.V.P.) which defrayed in part the cost of this investigation.

REFERENCES

Bain, J. A. & Rusch, H. P. (1944). *J. biol. Chem.* **153**, 659.
Beaven, G. H., Holiday, E. R. & Johnson, E. A. (1955). In *The Nucleic Acids*, vol. 1, p. 493. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
Brown, A. H. (1946). *Arch. Biochem.* **11**, 269.
Caspersson, T. & Thorell, B. (1942). *Acta physiol. scand.* **4**, 97.
Ceriotti, G. (1955). *J. biol. Chem.* **214**, 59.
Clavert, J., Mandel, P., Mandel, L. & Jacob, M. (1949). *C.R. Soc. Biol., Paris*, **143**, 539.
Dische, Z. (1930). *Mikrochemie*, **8**, 4.
Dounce, A. L. (1955). In *The Nucleic Acids*, vol. 2, p. 93. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Gerendas, M. & Matoltsy, A. G. (1948). *Hung. acta phys.* **1**, 124, 128.
Greenstein, J. P. & Thompson, J. W. (1943). *J. nat. Cancer Inst.* **4**, 275.
Hamoir, G. (1951*a*). *Biochem. J.* **48**, 146.
Hamoir, G. (1951*b*). *Biochem. J.* **50**, 140.
Hasselbach, W. & Schneider, G. (1951). *Biochem. Z.* **321**, 462.
Hendler, R. W. (1956). *J. biol. Chem.* **223**, 831.
Loftfield, R. B. (1957). *Progr. Biophys. Biophysical Chem.* **8**, 347.
Magasanik, B. & Chargaff, E. (1951). *Biochim. biophys. Acta*, **7**, 396.
Markham, R. (1955). In *Modern Methods of Plant Analysis*, vol. 4, p. 266. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer-Verlag.
Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 558, 565.
Mihalyi, E., Brodley, D. F. & Knoller, M. I. (1957). *J. Amer. chem. Soc.* **79**, 6387.
Mihalyi, E., Laki, K. & Knoller, M. I. (1957). *Arch. Biochem. Biophys.* **68**, 130.
Needham, D. M. & Cawkwell, J. M. (1957). *Biochem. J.* **65**, 540.
Perry, S. V. (1952). *Biochim. biophys. Acta*, **8**, 499.
Perry, S. V. (1953). *Biochem. J.* **55**, 114.
Perry, S. V. & Corsi, A. (1958). *Biochem. J.* **68**, 5.
Perry, S. V. & Grey, T. C. (1956). *Biochem. J.* **64**, 184.
Perry, S. V. & Zydowo, M. (1958). *Proc. 4th Int. Congr. Biochem., Vienna*, p. 83.
Perry, S. V. & Zydowo, M. (1959). *Biochem. J.* **71**, 220.
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
Porter, K. R. & Palade, G. E. (1957). *J. biophys. biochem. Cytol.* **3**, 269.
Roth, J S. & Milstein, S. W. (1952). *J. biol. Chem.* **196**, 489.
Schmidt, C. G. & Schlieff, H. (1956). *Z. ges. exp. Med.* **127**, 53.
Sheng, P-K. & Tsao, T.-C. (1954). *Acta physiol. sinica*, **19**, 203.
Szent-Györgyi, A. G., Mazia, D. & Szent-Györgyi, A. (1955). *Biochim. biophys. Acta*, **16**, 339.
Tarr, H. L. A. (1958). *Annu. Rev. Biochem.* **27**, 223.
Tissières, A. & Watson, J. D. (1958). *Nature, Lond.*, **182**, 778.
Volkin, E. & Cohn, W. E. (1953). *J. biol. Chem.* **205**, 767.
Warburg, O. & Christian, W. (1941–42). *Biochem. Z.* **310**, 384.
Woodward, G. E. (1944). *J. biol. Chem.* **156**, 143.
Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

Two-Way Separation of Amino Acids and Other Ninhydrin-Reacting Substances by High-Voltage Electrophoresis Followed by Paper Chromatography

By MARY L. EFRON
Medical Unit, University College Hospital Medical School, London, W.C. 1

(*Received* 27 *January* 1959)

One-way separation of amino acids by electrophoresis at high voltage is now a well-established procedure (Gross, 1955). With the large number of amino acids often found in biological fluids, it is, however, very difficult to obtain satisfactory separation without overlap of spots.

Several investigators have used techniques for the separation of amino acids and other substances by a combination of paper electrophoresis and chromatography. Durrum (1950, 1951) developed the so-called 'strip-transfer' method. This consisted in cutting out the strip of paper on which chromatography had previously been carried out, blotting the spots on to a second sheet and carrying out ionophoresis at right angles. This technique was further elaborated by Honegger (1956). Consden & Glynn (1955) described another two-dimensional method in which chromatography followed by ionophoresis on the same paper was used.

All these methods utilize low-voltage electrophoresis, which does not give a good separation of the neutral amino acids. Since this paper was written, it has come to the author's attention that Dixon, Kauffman & Neurath (1958) have described the two-way separation of amino acids by using a combination of electrophoresis at 1500v in one direction and chromatography in the other. They found this method more satisfactory than previously-used chromatographic methods. By modifying the apparatus described by Gross (1956) for one-way high-voltage electrophoresis in such a way that a square paper can be used, amino acids can be separated by high-voltage electrophoresis along one edge of the paper, and chromatography subsequently carried out in the other direction. The two-way separation thus obtained has several advantages over previously described chromatographic methods, especially with biological fluids.

METHODS

The apparatus described by Gross (1956) is used, except that the cooling plates are 16¾ in. × 17 in. Whatman no. 4 filter paper, 16½ in. × 18½ in., is wetted with formic acid–acetic acid buffer, pH 2 (see Gross, 1956), and the edges are placed between paper wicks fed from polythene beakers containing the electrodes, partially immersed in the same buffer. The solution containing the amino acid mixture is applied to the lower right-hand corner of the paper near the anode. If the quantity of solution to be examined is 25 μl. or less in volume, it can be applied directly to the wet paper. If the volume is greater than 25 μl. it should be applied to the dry paper in 25 μl. portions, the paper being dried in a hot-air stream between applications. The sheet is then dipped in buffer, care being taken not to wet the lower right-hand corner where the specimen has been applied. After being blotted to remove excess of moisture the paper is placed between the wicks and the dry corner then sprayed lightly with the same buffer. In this way elution of the applied amino acids is avoided during the wetting of the paper. With this apparatus the electrophoresis run can be carried out on two papers simultaneously, provided that each paper is placed between separate wicks and that the two papers are separated from each other by a sheet of polythene 0·005 in. thick. In this case a current of approximately 200 mA is produced when 3·5 kv is applied to the electrodes. The positions of the spots and the time required for the separation are the same as with a single sheet.

A potential difference of 3·5 kv is then applied to the electrodes, producing a current of approximately 120 mA. The amino acids travel towards the cathode, i.e. from right to left. A rapid and reproducible one-way separation of amino acids in urine, sweat or standard solution is obtained in 30–35 min. Electrophoresis of plasma samples (625 μl.) requires about 50 min., and spinal fluid (625 μl.) 60 min., twice as long as with the smaller-volume urine specimens. The paper is dried either in an oven or at room temperature. It is then ready for chromatography in the right-angle direction, i.e. from bottom to top.

If square cooling plates are not available, the apparatus similar to that described by Gross (1956), which is now available commercially (Locarte Co., London; Shandon Automation and Electronics Ltd., London), can be used. In this case the amino acid mixture is applied to the lower right-hand corner of a standard Whatman no. 4 paper, 18½ in. × 22 in., and the top 4½ in. of the paper moistened with buffer. Only this wet strip (4½ in. × 22 in.) is placed under the cooling plates (6 in. × 19½ in.); the remainder of the sheet is kept dry, so that no current passes through it. Electrophoresis is then carried out in the usual way, producing a good one-way separation of amino acids along the wet strip. The paper is then dried and is ready for chromatography from bottom to top. This method is less convenient than using square cooling plates, but the results are quite satisfactory.

In this Laboratory the standard descending paper-chromatographic technique is used, with a lutidine–water mixture (2:1, v/v) as the solvent. This requires 7–8 hr. for a sheet of this size, but the paper can be left overnight if the amount of solvent is limited to 30 ml./paper.

After being dried in an oven the paper is sprayed with a 0·1% (w/v) solution of ninhydrin in butanol, and then heated at 60° for 2 hr. or 100° for 1–2 min.

Preparation of biological fluids for electrophoresis

Urine. Urine can be applied directly to the wet paper, provided that it is free from protein. As a small amount of protein causes considerable streaking of amino acids during high-voltage electrophoresis, it is necessary to deproteinize any specimens giving a positive test for protein. This can rapidly be accomplished by adding 50 mg. of crystalline

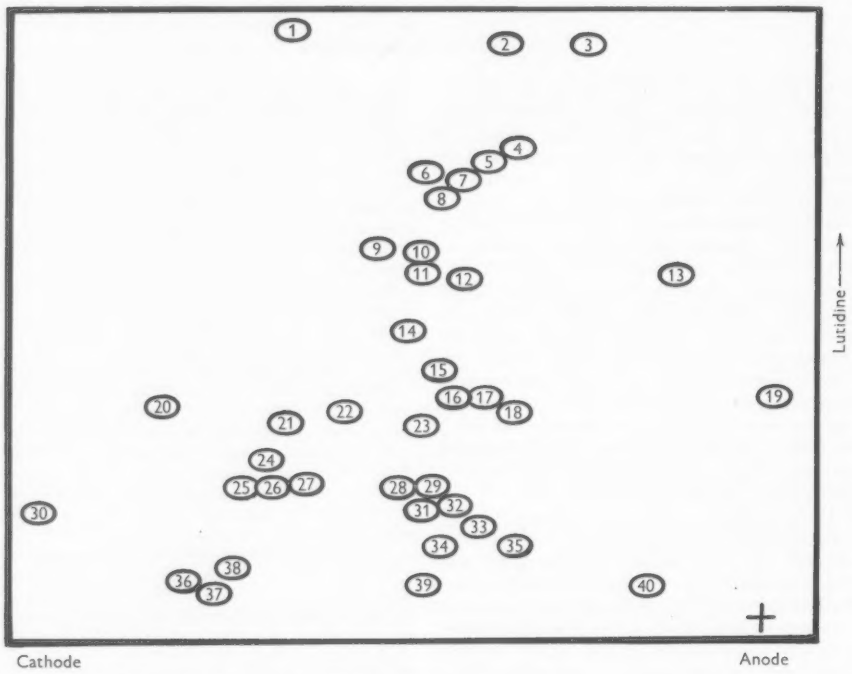


Fig. 1. Map of the location of amino acids and other ninhydrin-reacting substances. This shows the positions of spots, not tracings of their outlines. Each substance (5 μg.) in standard solution was applied at the lower right-hand corner (+) near the anode. Electrophoretic separation was from right to left, toward the cathode, a pH 2 buffer being used. A potential difference of 3·5 kv was applied, developing a current of 100–120 ma for 30 min. Lutidine was then run from bottom to top. The identifications are:

1. Tyramine
2. α-Amino-octanoic acid
3. 3:5-Di-iodotyrosine
4. Tryptophan
5. Tyrosine
6. Leucine
7. Phenylalanine
8. Methionine
9. Glucosamine
10. Norvaline
11. Valine
12. Methionine sulphone
13. Taurine
14. α-Aminobutyric acid
15. Threonine
16. Proline
17. Hydroxyproline
18. Methionine sulphoxide
19. Cysteic acid
20. Ethanolamine
21. β-Amino*iso*butyric acid
22. Alanine
23. Serine
24. Histidine; 1- and 3-Methylhistidine
25. β-Alanine
26. γ-Aminobutyric acid
27. Glycine
28. Homocystine
29. Glutamine
30. Putrescine
31. Asparagine
32. Glutamic acid; citrulline
33. Aspartic acid
34. Cystathionine
35. Glutathione
36. Ornithine
37. Lysine
38. Arginine
39. Argininosuccinic acid
40. Phosphoethanolamine

picric acid to 2 ml. of urine, centrifuging for 3 min. at 2500 rev./min. and decanting the supernatant. If this supernatant is clear yellow, the specimen has been adequately deproteinized. In an occasional specimen with heavy proteinuria the supernatant may still be cloudy. In this case an additional 50 mg. of picric acid should be added and the mixture centrifuged again. This will usually produce a clear supernatant. Picric acid does not interfere with electrophoresis, as it moves towards the anode when a buffer (pH 2) is used, whereas the amino acids move towards the cathode. A volume of urine containing 250 μg. of total nitrogen is the standard quantity used in this Laboratory for routine purposes. If overloading is desired, however, 5–10 times this amount can be used without desalting. Desalting is accomplished automatically during the electrophoretic separation and there is therefore no interference by urinary salts with the subsequent chromatography. With such an overloaded urine the electrophoresis must be continued for 50 min.

Plasma. Plasma can also be deproteinized with picric acid and run without further preparation. Most 2 ml. plasma samples are adequately deproteinized by the addition of 150 mg. of picric acid. An occasional sample, particularly one containing haemolysed red cells, may require the addition of a further 50 mg. of picric acid to produce a clear supernatant. Approximately 1·3 ml. of protein-free plasma is recovered from the initial 2 ml. sample. Of this volume, 625 μl. has been used as the standard quantity for routine study. It can be evaporated to a volume of 25–50 μl. before being applied to the paper, or can be applied in 25 μl. portions.

Spinal fluid. Spinal fluid is deproteinized by the addition of 25 mg. of picric acid to 1 ml. of fluid. As with plasma, desalting is unnecessary. The standard quantity for routine study is 625 μl.

Sweat. Sweat is treated in the same way as spinal fluid. The supernatant in this case, though adequately deproteinized, never becomes completely clear. The standard quantity for study is 125 μl.

RESULTS

A map of the positions taken by 43 ninhydrin-reacting substances in pure solution is reproduced in Fig. 1. Fig. 2 is a tracing of the spots due to ninhydrin-reacting substances present in the urine of a patient with typical Fanconi syndrome and gross amino aciduria. It can be seen that this method produces compact, round spots. Because of the short time required for electrophoretic separation, and the absence of salt interference, there is very little diffusion of the amino acid spots. Figs. 3 and 4 are tracings of the spots due to

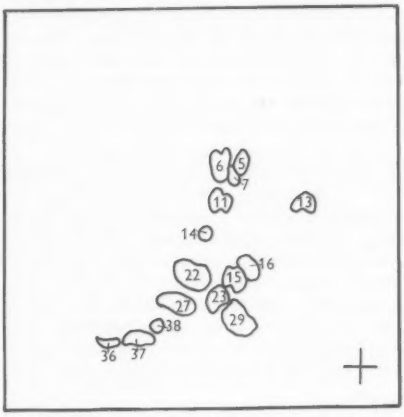


Fig. 3. Ninhydrin-reacting substances in normal plasma. Deproteinized plasma (625 μl.) is applied. For numbering of spots, see Fig. 1.

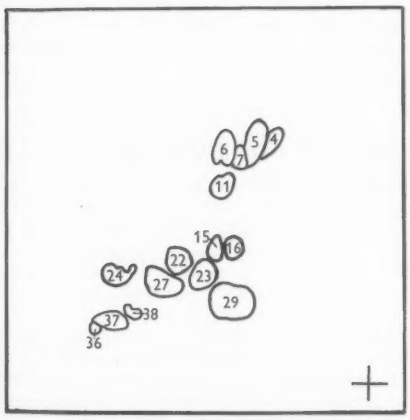


Fig. 2. Tracing of the spots in urine of a patient with Fanconi syndrome. An amount of urine equivalent to 250 μg. of nitrogen was applied. Spot identification as in Fig. 1.

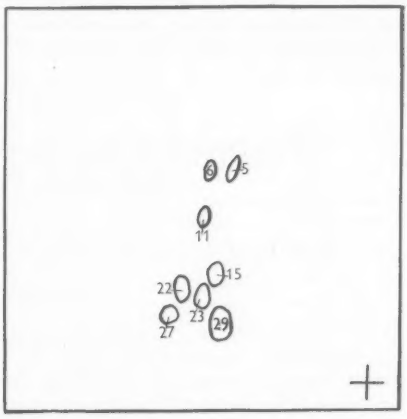


Fig. 4. Ninhydrin-reacting substances in normal cerebrospinal fluid. Deproteinized cerebrospinal fluid (625 μl.) is applied. For numbering of spots, see Fig. 1.

ninhydrin-reacting substances present in a normal plasma and in normal spinal fluid, prepared as described above.

DISCUSSION

The combined ionophoretic–chromatographic technique has certain advantages over conventional two-way chromatography. This method is rapid. Time is saved by: (*a*) replacing the 24 hr. first run in phenol with high-voltage electrophoresis taking 1½ hr.; (*b*) eliminating the need for desalting; (*c*) for protein-containing fluids (plasma etc.), avoiding the tedious process of ultrafiltration, which is replaced by a simple deproteinization with picric acid followed by centrifuging. The entire operation can be completed in 9–10 hr. instead of the 48 hr. required by two-way chromatography.

There is better separation of certain amino acids. For example, methionine separates from leucine, whereas in the phenol–lutidine chromatogram these spots overlap, and oxidation is necessary to detect the presence of methionine (Dent, 1947). Asparagine produces a distinct spot and does not overlap glycine. Cystathionine, argininosuccinic acid and phosphoethanolamine are more distinctly separated from each other. β-Amino*iso*butyric acid separates well from histidine. On the other hand, glutamine and glutamic acid are not so well separated as they are by two-way chromatography, and the 1- and 3-methylhistidines overlap histidine.

The spots are rounder and more compact than with two-way chromatographic techniques. The electrophoretic separation allows very little diffusion of the spots, because of the short time required to traverse a given distance. As a result of the automatic desalting during electrophoresis, the final chromatogram shows little distortion of the spots. Another advantage is that volatile substances such as ethanolamine are often found in pathological urines when not detected in the same specimen by routine chromatography.

SUMMARY

1. A technique for two-way separation of amino acids and other ninhydrin-reacting substances by high-voltage paper electrophoresis followed by paper chromatography is described, which gives more rapid separation than two-way paper-chromatographic methods.

2. The method is particularly useful for the study of plasma and spinal fluid, because these fluids can be deproteinized in a few minutes with picric acid, thus eliminating the tedious process of ultrafiltration. Moreover, desalting is not necessary, as it is automatically accomplished during the electrophoresis run.

3. A map is presented showing the positions taken by 43 ninhydrin-reacting substances when separated by this method.

The author is indebted to Professor C. E. Dent and to Mr R. G. Westall for constant advice and encouragement, to Dr D. C. Cusworth for help with amino acid chromatography, and to Mr C. E. Glover and Mr G. Marshall for construction and maintenance of apparatus.

REFERENCES

Consden, R. & Glynn, L. E. (1955). *Lancet*, **1**, 943.
Dent, C. E. (1947). *Biochem. J.* **41**, 240.
Dixon, G. H., Kauffman, D. L. & Neurath, H. (1958). *J. biol. Chem.* **233**, 1373.
Durrum, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
Durrum, E. L. (1951). *J. Colloid Sci.* **6**, 274.
Gross, D. (1955). *Nature, Lond.*, **176**, 72.
Gross, D. (1956). *Nature, Lond.*, **178**, 29.
Honegger, C. G. (1956). *Helv. chimica acta*, **39**, 1671.

The Isolation and Characterization of Acetylcholine-Containing Particles from Brain

By V. P. WHITTAKER
Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(*Received* 28 *January* 1959)

Chemical transmission at nerve endings is now being increasingly discussed in terms of the 'synaptic vesicle' theory (del Castillo & Katz, 1956; Eccles, 1957; Hebb, 1957). Synaptic vesicles are small bodies about 0·05 μ in diameter observable in electron micrographs of thin sections of nervous tissue and concentrated in large numbers at many different kinds of nerve endings (de Robertis & Bennett, 1955; de Robertis & Franchi, 1956; Robertson, 1956; Palay, 1956; Luft, 1956); they are assumed to contain chemical-transmitter substances (e.g. acetylcholine in cholinergic neurones) which are released in response to the arrival of a nerve impulse.

This theory provides a reasonable explanation of a number of facts about chemical-transmitter substances. The 'quantized' release of acetylcholine at neuromuscular junctions detected electrophysiologically by del Castillo & Katz (1956) might be brought about by the spontaneous or synchronized bursting of such vesicles. The coexistence in the same tissue of transmitter substances and enzymes which rapidly destroy them *in vitro*, also of receptor sites, implies the existence of permeability barriers which might be provided by the walls of the vesicles. Acetylcholine and other putative transmitter substances are known to exist in nervous tissue partly in a 'bound' form. The properties of 'bound' acetylcholine suggest that it is acetylcholine held in some kind of subcellular structure analogous to or perhaps identical with mitochondria (Bodian, 1942; Feldberg, 1945; Braganca & Quastel, 1952; Brodkin & Elliott, 1953; Stone, 1955).

Hebb & Whittaker (1958) have recently described the isolation of a subcellular fraction of brain tissue distinct from mitochondria, nuclei and microsomes, which contains the bound acetylcholine and choline acetylase. It seemed possible that this fraction might represent a preparation of synaptic vesicles. Accordingly, the fraction has been further studied biochemically and morphologically from this point of view. It has been found to consist largely of particles having the same size range as that reported for synaptic vesicles and distinct in size, structure and enzyme content from mitochondria. The distributions of 5-hydroxytryptamine, another compound for which a transmitter action in the central nervous system has been proposed, and of two lysosome enzymes, acid phosphatase and β-glucuronidase, were compared with that of acetylcholine in order to throw light on the degree of homogeneity of the acetylcholine-containing fraction. The distribution of 5-hydroxytryptamine resembled that of acetylcholine. The two lysosome enzymes gave conflicting results and suggested that the lysosome hypothesis cannot be applied to brain without modification. Finally, the conditions influencing the release of acetylcholine from the particles have been studied in an attempt to find out how far their properties can account for the phenomenon of bound acetylcholine and to elucidate the mechanism of acetylcholine release *in vivo*.

Preliminary accounts of part of this work have already been given (Whittaker, 1958*a*, *b*, 1959).

METHODS

Estimation of pharmacologically active substances and enzymes

Free and bound acetylcholine. Assays of acetylcholine were carried out on the rectus abdominis muscle of the frog (Chang & Gaddum, 1933). To determine total acetylcholine, the bound ester was first released by treating the sample at pH 4 and 100° for 10 min. Since shaking with ether also releases all the bound acetylcholine, this method was also used in some experiments. Free acetylcholine was determined by assaying samples directly without prior treatment. The difference between the total and free acetylcholine content of a sample was taken to represent the bound acetylcholine therein.

The presence of sucrose in the organ bath was found to modify the response of the muscle to acetylcholine; this was allowed for by the addition of sucrose to the standards. High concentrations of sucrose caused a contraction in the absence of acetylcholine; this made the assay of low concentrations of acetylcholine in such solutions difficult, but the difficulty could be avoided when the acetylcholine content of particles was being assayed by resuspending the particles in 0·32 M-sucrose before releasing acetylcholine by acid treatment.

Assay of 5-hydroxytryptamine. This was carried out by the rat-fundus method of Vane (1957), atropine (0·2 mg./ml.) being added to the medium to block acetylcholine.

Succinic dehydrogenase. This was estimated manometrically at 37° according to Hogeboom, Claude & Hotchkiss (1946), as modified by W. N. Aldridge & M. K. Johnson (personal communication). The hydrogen acceptor was 0·2 ml. of 0·25 M-$K_3Fe(CN)_6$ in 0·023 M-$NaHCO_3$, placed in the side bulbs of Warburg flasks. The main compartment contained enzyme in 1·0 ml. of 0·32 M-sucrose and 1·8 ml. of a medium consisting of 0·14 ml. of 2 M-$MgCl_2$, 0·5 ml. of 0·1 M-ethylenediaminetetra-acetic acid, 1·0 ml. of M-phosphoric acid, 6·0 ml. of 0·3 M-succinic acid and 4·0 ml. of 0·27 M-$NaHCO_3$ in a final volume of 30 ml. The other ingredients of the medium were adjusted to pH 7·4 before the $NaHCO_3$ solution was added. The flasks were gassed with $CO_2 + N_2$ (5:95, v/v), and after temperature equilibration the reaction was started by tipping in the side-bulb contents. The rate of displacement of CO_2 from the bicarbonate buffer by the H^+ ions formed during the reduction was taken as a measure of the enzyme activity, 1 unit (u.) of which = 1 μl. of CO_2/hr. Measurements were average of duplicate determinations made during the first 6 min. of the reaction. Flasks with no enzyme were included as controls, but the correction for non-enzymic CO_2 evolution was usually negligible.

Acid phosphatase. This was estimated in 0·1 M-citrate buffer, pH 4·9, using 0·05 M-phenylphosphate as substrate as described by Cohn & Richter (1956), or in a few experiments by the method of Berthet & de Duve (1951), with sodium β-glycerophosphate as substrate after activating the enzyme by mechanical disruption of the particles in a Mickle shaker. The use of the first method was considered to be legitimate owing to the absence of glucose 6-phosphatase from brain and was preferred to the other as more sensitive, enabling fractions to be fully activated by dilution. The two methods gave similar results. Activities are expressed as μg. of P liberated/hr.

β-Glucuronidase. This was estimated by the method of Kerr & Levvy (1951). Activity is expressed in glucuronide units (G.U.) as defined by these authors.

Cholinesterase. 1 unit (u.) is that amount of enzyme which releases 1 μl. of CO_2/min. from 10 mM-acetylcholine in CO_2–$NaHCO_3$ buffer, pH 7·4 at 37°.

Expression of activities. Activities of fractions are expressed/ml. fraction or/volume of fraction equivalent to 1 g. of fresh brain tissue, as percentages of the total recovered activity, or as relative specific activities. This last quantity is defined as the activity of the fraction expressed as a percentage of the total recovered activity divided by the nitrogen content of the fraction as a percentage of the total recovered nitrogen.

Nitrogen estimations. These were carried out by the micro-Kjeldahl method.

Electron microscopy. This was carried out on sections (approximately 200 Å thick) of fractions fixed and stained in OsO_4 and embedded in methyl methacrylate resin. The sections were cut by Dr K. M. Smith and the electron micrographs were taken by Mr G. J. Hills. Fractions were diluted with an equal volume of 2% (w/v) OsO_4 in 0·32 M-sucrose, adjusted to pH 7·4 with NaOH and centrifuged at 8×10^5 *g*-min., and the pellet was embedded after storage overnight at 0–2°.

Fractionation of brain tissue

Preparation of primary fractions. The preparation and fractionation of homogenates of rabbit and guinea-pig brain in 0·32 M-sucrose containing 50 μM-eserine into 'nuclear' (P_1), 'crude mitochondrial' (P_2), 'microsomal' (P_3) and 'cytoplasmic' (S) fractions were carried out as described by Hebb & Whittaker (1958), with some modifications. Thus the microsomal and cytoplasmic fractions were often not separated but studied as a combined fraction (S_2). In most experiments, a glass and Perspex homogenizer (rotating at 840 rev./min.) with a difference in diameter of 0·025 mm. between pestle and mortar (W. N. Aldridge, personal communication) was used instead of the all-glass homogenizer, with a somewhat greater and variable clearance, previously used. Sometimes eserine was omitted from the sucrose; under these circumstances the free acetylcholine of the supernatant fraction was destroyed and the total acetylcholine content of the homogenate was lower by a like amount. In some experiments, the yield of acetylcholine particles in the P_2 fraction was improved by washing the P_1 fraction twice with sucrose and by sedimenting the P_2 fraction in an integrated field of 8 instead of $4·5 \times 10^5$ *g*-min. (The gravitational effect of centrifuging is expressed in *g*-min., i.e. the product of the force and the time, in minutes, of spinning.) Variations in the fractionation procedure were controlled by phase-contrast microscopy and by determinations of bound acetylcholine and succinic dehydrogenase (SDH). A typical result is given in Fig. 1, from which it will be seen that the two activities parallel each other closely in their distribution in the primary fractions. Since SDH is generally considered to

Fig. 1. Distribution of bound acetylcholine (■) and succinic dehydrogenase (SDH) (□), expressed as a percentage of the total recovered activity between the twice-washed nuclear fraction (P_1), crude mitochondrial fraction (P_2), and microsomal plus cytoplasmic fraction (S_2), from guinea-pig brain. The P_2 fraction was separated at 8×10^5 *g*-min. Activity of original homogenate: acetylcholine, 11·7 μm-moles/g. of fresh tissue; SDH, 9900 u./g. of fresh tissue. Recovery of activity in fractions: acetylcholine, 91%; SDH, 80%. The results are the average of four experiments.

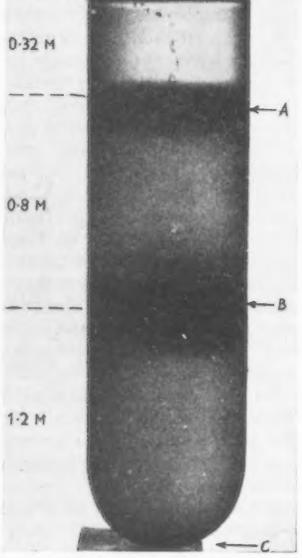


Fig. 2. Appearance of a tube showing the three fractions *A*, *B* and *C* after centrifuging 1 ml. of P_2 from guinea-pig brain into a sucrose density gradient consisting of 2 ml. of 0·8 M- and 2 ml. of 1·2 M-sucrose at $7·5 \times 10^5$ *g*-min. in the Spinco SW 39 head. The tube was photographed in transmitted light.

be exclusively located in mitochondria, it is clear in confirmation of previous findings that the bound acetylcholine is located in particles which have sedimentation properties broadly similar to these of mitochondria and that the activities of P_1 and S_2 are due to contamination of these fractions by P_2.

Subfractionation of the P_2 fraction. Subfractionation of P_2 by equilibrium centrifuging in density gradients was carried out as described by Hebb & Whittaker (1958), the SW 39 head of the Spinco Model L preparative ultracentrifuge being used. In the simplified procedure used, the P_2 fraction was resolved into three main subfractions, *A*, *B* and *C*, by centrifuging 1 ml. quantities into a density gradient consisting of 2 ml. of 1·2M- and 2 ml. of 0·8M-sucrose in an integrated field of about $7{\cdot}5 \times 10^6$ *g*-min. (Fig. 2). *A* was white material of density intermediate between that of 0·32M- and 0·8M-sucrose, largely inactive and consisting mainly of the largest (2–5 μ) particles in P_2; *B* was greyish white material of density intermediate between that of 0·8M- and 1·2M-sucrose, containing most of the acetylcholine and consisting of the smallest particles in P_2; while *C* was a tan-coloured precipitate of material more dense than 1·2M-sucrose, containing most of the SDH, little acetylcholine and large numbers of typical rod-shaped mitochondria. The sub-fractions were separated in the cutter by sections through the relatively clear zones of sucrose between them. The particles contained in them were returned to 0·32M-sucrose for assay and storage by diluting with water to bring the sucrose concentration to 0·32M, centrifuging in an integrated field of 8×10^5 *g*-min. or more and resuspending the precipitate in 0·32M-sucrose. A sample of the parent fraction, made M with solid sucrose, was similarly diluted, centrifuged and resuspended as a control. In contrast with some earlier results, this process of dilution and resuspension could be achieved with little or no loss of bound acetylcholine, provided that the particles were not exposed to hyperosmotic conditions for too long.

The use of the SW 39 head suffered from the disadvantage of providing only very small quantities of material in each run. Accordingly in some experiments the differences in density were utilized for larger-scale separations in the Spinco no. 40 head. Centrifuging in a density gradient is not conveniently carried out in this head as, owing to the fixed inclination of the tubes, boundaries change position and layers overlap during runs. However, it was possible to effect separations by first centrifuging samples of P_2 into a fairly large volume of 0·8M-sucrose, leaving the relatively large light particles corresponding to subfraction *A* at the top of the tube, and then separating the mixture of denser particles corresponding to subfractions *B* and *C* in a second run with 1·2M-sucrose. The procedure is illustrated diagrammatically in Fig. 3 and analyses of the fractions from one of these experiments are presented in Table 1. It will be seen that although fraction *A* retained appreciable amounts of acetylcholine and SDH, most of the recovered acetylcholine was present in fraction *B* and most of the recovered SDH was present in fraction *C*. The relative

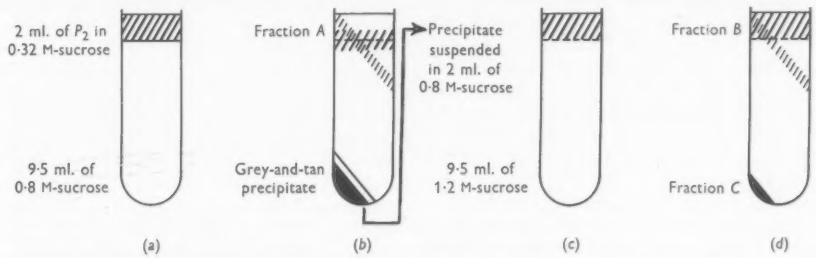


Fig. 3. Appearance of centrifuge tube (*a*) before, (*b*) after centrifuging 2 ml. of P_2 from rabbit brain into 9·5 ml. of 0·8M-sucrose in the no. 40 head of the Spinco Model L preparative ultracentrifuge. Fraction *A* was collected, the intermediate clear zone discarded and the precipitate resuspended in 2 ml. of its supernatant, transferred to a second tube containing 9·5 ml. of 1·2M-sucrose shown (*c*) before and (*d*) after centrifuging similarly. Corresponding fractions from two tubes were pooled for analysis (Table 1).

Table 1. *Acetylcholine, succinic dehydrogenase and nitrogen content of subfractions of P_2*

For details and for definition of relative specific activity (RSA) see Methods.

		Acetylcholine		SDH	
Fraction	N as % recovered N	Recovered activity (%)	RSA	Recovered activity (%)	RSA
A	53	33	0·62	13	0·25
B	30	67	2·1	16	0·53
C	17	0	0	71	4·2
Recovery*	76	96	—	58	—

* As percentage of P_2 (from rabbit brain). Composition: N, 3·78 mg./ml.; acetylcholine, 2·9 μm-moles/ml.; SDH, 5560 u./ml.

specific activities (cols. 4 and 6) also emphasize the different distribution of the two activities in the fractions.

When fraction *B*, thus prepared, was refractionated in a density gradient consisting of layers of 1·2 M-, M- and 0·8 M-sucrose, with the SW 39 head, most of the particles migrated as a single dense white band to a position just above the 1·2 M layer, indicating a density equivalent to that of M-sucrose, i.e. of about 1·15 g./ml. at 20°. Most of the acetylcholine remained in association with this band. A small proportion of the particles and of the acetylcholine was recovered in two other faint zones in adjacent layers, indicating that the particles vary no more in density than by about ±3% of the mean.

RESULTS

Electron microscopy of subfractions

Electron micrographs of fractions *B* and *C* (from guinea-pig brain) are shown in Plate 4 (2, 3). Most of the particles in fraction *C* [Plate 4 (3)] having the high SDH content are typical mitochondria showing the characteristic structure of limiting membrane and internal cristae mitochondriales. Their appearance closely resembles that of rat-liver mitochondria isolated from sucrose homogenates (Novikoff, 1957); there is internal vesication; some mitochondria are swollen and have lost their cristae or these have shrunk to one pole. Fragments of mitochondrial membranes and some smaller particles can also be seen. Their density is similar to that found for liver mitochondria by Kuff & Schneider (1954). By contrast, in fraction *B* [Plate 4 (2)], rich in acetylcholine, mitochondria are almost completely absent, but there are large numbers of smaller particles, ranging in diameter from 0·3 down to about 0·02 μ. These on section appear as simple vesicles. There are also other structures which may be fragments of mitochondrial membranes and portions of the endoplasmic reticulum. A few of the larger particles resemble rat-liver lysosomes in appearance (Novikoff, 1957). It seems reasonable to assume that some at least of the particles seen in Plate 4 (2) are those containing acetylcholine. Electron micrographs of preparations of fraction *B* in which the separation of SDH activity was less complete showed considerable numbers of the larger particles with the internal structure of mitochondria.

Counts of particle size were made of several micrographs each containing several hundred particles. The logarithms of particle diameters were found to be normally distributed (Fig. 4), the median diameter of particles of fraction *B* being 0·065 μ, λ 0·24, and that of particles of fraction *C* being 0·26 μ, λ 0·22, where λ is the standard deviation of the logarithm (to base 10) of the diameters. Over 60% of the particles in fraction *B* fell into the size range (0·02–0·08 μ) reported for synaptic vesicles (Palay, 1956). The distribution of particle diameters in fraction *C* was not quite lognormal, but could be made so if it were corrected on the assumption that fraction *C* contained 10% of particles from fraction *B*.

The appearance of the particles in fraction *A* as seen with phase contrast is shown in Plate 4 (1). Their nature has not yet been elucidated. They contain little ribonucleic acid or deoxyribonucleic acid and do not react like nuclei to nuclear stains; they are therefore probably not of nuclear or microsomal origin. They may be small enucleated cells, possibly derived from glial tissue.

Distribution of active substances other than acetylcholine in the density gradient

In order to gain some idea of the degree of homogeneity of the acetylcholine-particle preparation, the distribution of some other active substances in the density gradient was compared with that of acetylcholine. Particular interest attached to 5-hydroxytryptamine (5-HT). This has been postulated to have a transmitter action in the central nervous system and has been stated to be located in mitochondria (Walaszek & Abood, 1957). Because the stability of the acetylcholine-containing particles bore some resemblance to that of lysosomes (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955), the distribution of two lysosomal enzymes, acid phosphatase and β-glucuronidase, was studied. There is evidence that rat brain contains lysosome-like particles (Beaufay, Berleur & Doyen, 1957), but information about their sedimentation and other properties is lacking.

Distribution of 5-hydroxytryptamine in fractions. The 5-HT content of sucrose homogenates of guinea-pig brain was found to be between 0·9 and 1·9 μm-moles/g., which is near the values given by Correale (1957) and Walaszek & Abood (1957), if μmg. is read for μμg. in the latter paper. About 25–40% of the total 5-HT in the homogenate was

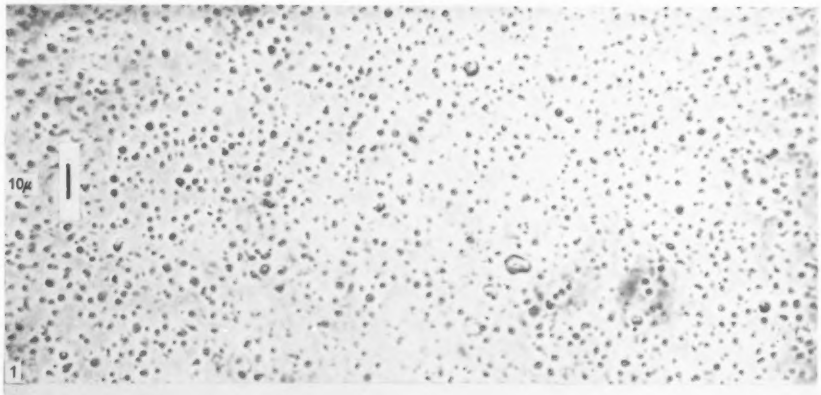
EXPLANATION OF PLATE 4

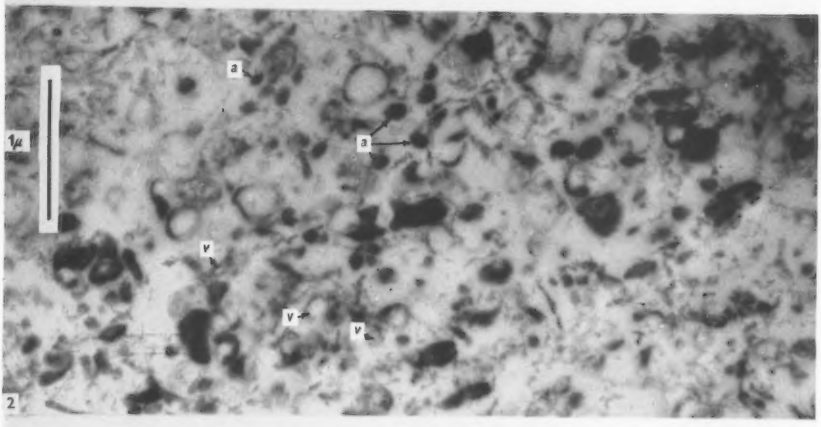
1. Phase-contrast micrograph of fraction *A* of guinea-pig brain. Wet preparation stained with osmium tetroxide.

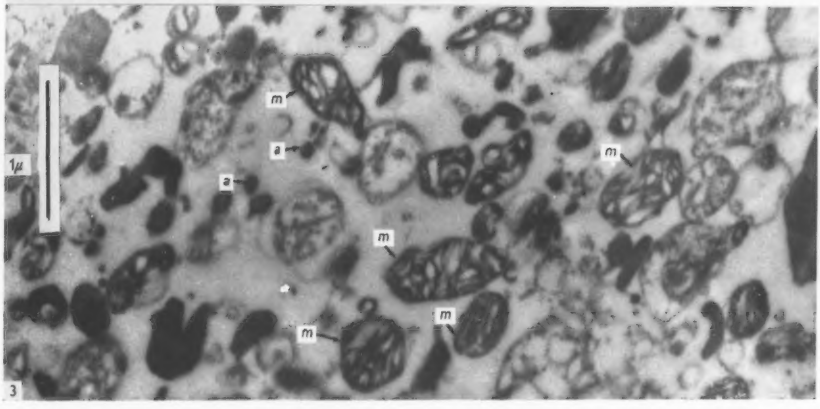
2. Electron micrograph of ultra-thin section of pellet (fixed and stained in osmium tetroxide) embedded in methyl methacrylate resin, from fraction *B* of guinea-pig brain. Large numbers of small particles (*a*), 0·02–0·3 μ in diameter, can be seen. Some appear in section as simple vesicles (*v*).

3. Electron micrograph prepared in the same way as (2) from fraction *C*. Many typical mitochondria (*m*) with lengths up to 1 μ can be seen. A few smaller bodies (*a*) similar to the larger particles in (2) are also present.

Fractions were prepared from the P_2 fraction by using the Spinco no. 40 head as described in the Methods section.







V. P. WHITTAKER—The isolation and characterization of acetylcholine-containing particles from brain

(*Facing p.* 698)

found to be in the 'free' form; this was recovered in the supernatant fraction. The remainder was mainly in the P_2 fraction. On subfractionating the latter in a density gradient using the SW 39 head, most of the activity was found to be in the *B* fraction, which also has a high acetylcholine content. The result of a typical experiment is given in Fig. 5. Particulate fractions, even after resuspension in fresh 0·32 M-sucrose, appeared to contain some free 5-HT. With P_2 this amounted to 7–35 % of the total 5-HT of the fraction. Since reasonably good recoveries of particulate 5-HT were attained on subfractionation, the particles could not have been losing 5-HT continuously during the preparation at 0°, and it was concluded that the free 5-HT in particulate fractions represented 5-HT liberated from the particles during the assay. In this respect 5-HT differs from acetylcholine, but the difference may be due to the higher temperature at which the 5-HT assays are carried out (37° for the rat fundus compared with 20–22° for the frog rectus).

Distribution of lysosome enzymes in fractions. Table 2 shows the distribution of acid phosphatase and β-glucuronidase in the various fractions and subfractions of a guinea-pig-brain homogenate. SDH and acetylcholine assays were carried out on the same fractions and are included for comparison. It will be seen that the two enzymes have different distributions in the density gradient, the distribution of acid phosphatase paralleling that of acetylcholine, while that of β-glucuronidase more closely resembles that of SDH.

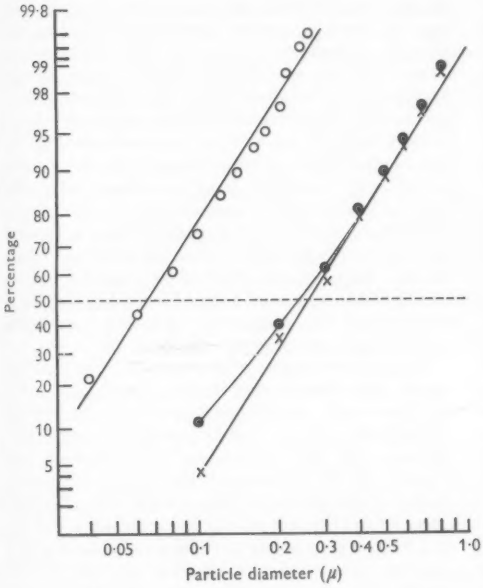


Fig. 4

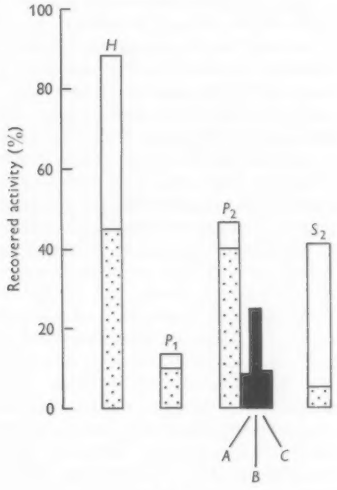


Fig. 5

Fig. 4. Lognormal distribution of particle size in subfractions *B* and *C* [Plate 4 (2 and 3)]. Total number of particles counted: *B*, 1126; *C*, 289. Ordinates, percentage of total population having indicated diameter and below, plotted on linear scale of probits; abscissae, particle diameters, plotted on logarithmic scale. ○, Subfraction *B*; ●, subfraction *C*; ×, subfraction *C* corrected for 10% contamination by subfraction *B*. It will be seen that the distribution of *B* and the corrected distribution of *C* lie close to the lognormal distributions indicated by the straight lines.

Fig. 5. Bound (dotted blocks) and free (white blocks) 5-hydroxytryptamine (5-HT) in a sucrose homogenate of guinea-pig brain (*H*) and in the P_1, P_2 and S_2 fractions derived from it, expressed as a percentage of the total recovered activity. The black blocks show the distribution of activity in the three subfractions (*A*, *B* and *C*) of P_2, prepared in the SW39 head. Total 5-HT content of H, 0·9 μm-mole/g. Recovery of free activity of *H* in S_2, 92%; recovery of bound activity of *H* in P_1, P_2 and S_2, 125%.

Table 2. *Distribution of acetylcholine, succinic dehydrogenase and lysosome enzymes in subcellular fractions of guinea-pig brain*

The figures are the activity expressed as a percentage of total recovered activity. *A*, *B* and *C* were obtained from P_2 by using the Spinco SW 39 head. The eserine-free sucrose homogenate of guinea-pig brain from which all the fractions were prepared had the following activities: acetylcholine, 12·8 μm-moles/g., SDH, 13 200 u./g., acid phosphatase (substrate, disodium phenylphosphate) 3·0 mg. of P/hr./g., β-glucuronidase, 285 u./g.

Fraction	Acetylcholine	SDH	Acid phosphatase	β-Glucuronidase
P_1	12	23	24	30
P_2	70	77	42	51
S_2	18	0	34	19
Recovery	100	74	113	80
A	19	0	26	14
B	62	26	59	30
C	19	74	15	56
Recovery	93	59	136	93

Factors influencing the release of particulate acetylcholine and 5-hydroxytryptamine: further evidence for 'labile' and 'stable' forms of bound acetylcholine and for an ionic component in the latter

Previous work (Brodkin & Elliott, 1953; Hebb & Whittaker, 1958) has indicated that about 50% of the particle-bound acetylcholine is in a labile form which is readily released by relatively mild treatments such as osmotic dilution, freezing and thawing, or dialysis. The rest is in a more stable form which requires treatment with hydrochloric acid, trichloroacetic acid or ether for release. The stability of this second fraction is attested to by numerous earlier observations (for references see Feldberg, 1945), which have shown that it is not released by repeated washing or grinding of particulate material and is resistant to cholinesterases.

Further investigation of the conditions under which acetylcholine is released was undertaken in an attempt to clarify the nature of the binding forces and the mechanism of acetylcholine release *in vivo*. Conditions which released the labile form only—ageing at 0°, osmotic dilution, freezing and thawing, shaking with glass Ballotini beads, treatment with cobra venom—are all those which might be expected to increase the permeability of subcellular particles without disrupting them completely, thus confirming earlier suggestions that the labile fraction represents free acetylcholine sequestered within an easily damaged membrane. Conditions which released both forms included incubation for 1 hr. at 37°, treatment with organic solvents and exposure to pH values of 4 or less; the first two and perhaps the last would be expected to cause extensive damage to subcellular structures, possibly by processes of autolysis or denaturation; in addition, the displacement of the stable form by H^+ ions might point to the presence of an ionic component in the binding.

Provided that the particles were not subjected to even mildly disruptive procedures they could be centrifuged and washed repeatedly or exposed to cholinesterases, certain drugs and inorganic ions with little or no loss of either fraction. Since released acetylcholine (or added free acetylcholine) was, by contrast, rapidly destroyed by the cholinesterase invariably present in the preparations, eserine (50 μM) was always added, either to the fraction being studied or to the original homogenate from which it was prepared, when the release of acetylcholine was to be followed directly by assay of the liberated ester. Some of the substances tested (e.g. inorganic ions) interfered with the assay; release was then measured by comparing the acetylcholine content of treated particles with that of a control sample after collecting them by centrifuging. Experiments were usually carried out on the P_2 fraction; the *B* fraction behaved similarly, except that the proportion of labile particulate acetylcholine was slightly smaller.

Freezing and thawing. Samples of P_2 were withdrawn after freezing and thawing varying numbers of times and centrifuged, and free and bound acetylcholine determined on precipitate and supernatant. All the particulate acetylcholine was found to be in the bound form, and all the supernatant in the free form. Recoveries of total acetylcholine from precipitate and supernatant were close to 100%. The freezing and thawing caused a progressive release of acetylcholine into the supernatant (Fig. 6*a*), but this was only about 50% complete after 20 freezings and thawings and was not thereafter much increased. After 30 freezings and thawings, the total acetylcholine of the preparation began to decline.

Osmotic pressure. Fig. 6*b* shows the release at 0° of acetylcholine from particles from P_2 suspended in sucrose solutions having approximately half, one-fifth and one-tenth the osmotic pressure of the cell sap. Acetylcholine is released relatively slowly in 0·16 M-, more rapidly in 0·08 M- and very rapidly in 0·032 M-sucrose, but the process again had an end point at about 50% of the total acetylcholine.

Moderately hyperosmotic conditions (2–3 times iso-osmotic), whether brought about by the addition of sucrose or sodium chloride to the suspension in 0·32 M-sucrose, had little or no effect on the particulate acetylcholine.

Shaking. Mechanical disintegration by shaking in the Mickle shaker at 50 c./sec. in the presence of glass Ballotini beads also released about 50 % of the particulate acetylcholine.

Hydrogen-ion concentration. The effect of H^+ ion concentration on the release of acetylcholine at 0° is shown in Fig. 6*c*. At pH 4 most of the acetylcholine is released in 20 min. even at 0°; above pH 4·5 the release is very much slower.

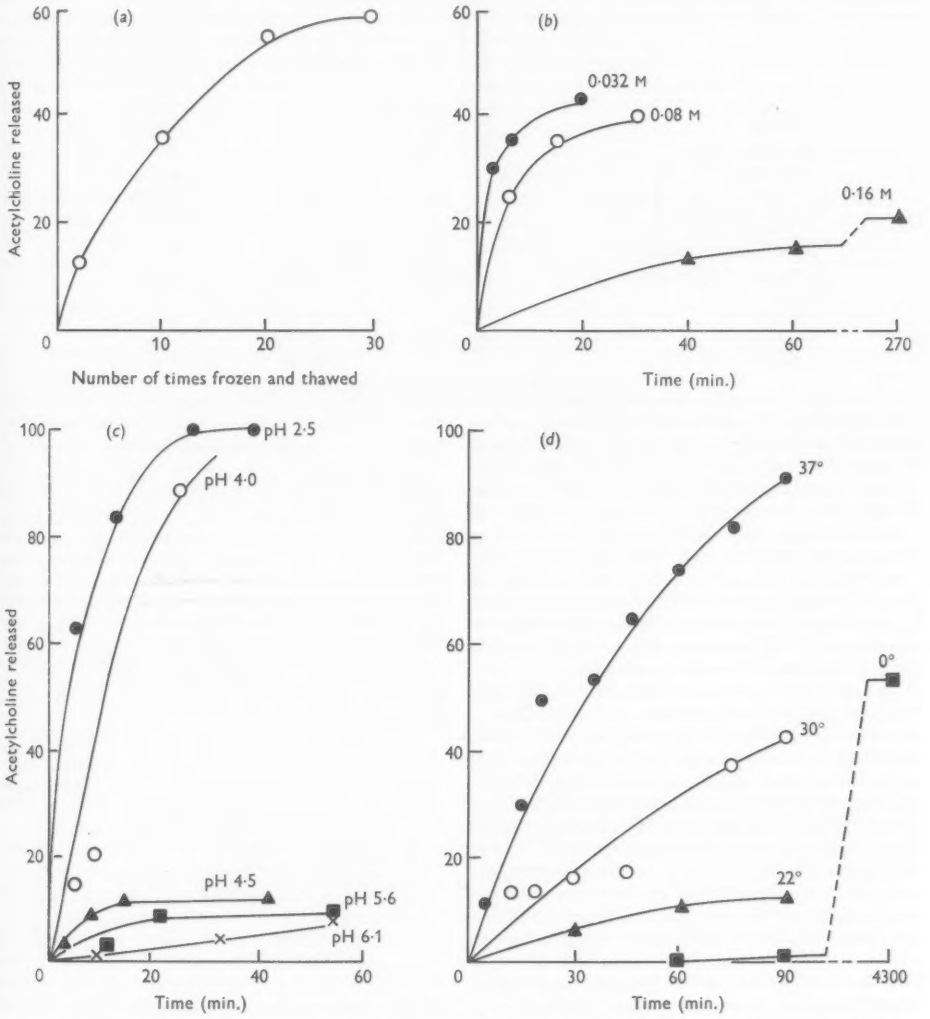


Fig. 6. Release of acetylcholine from P_2 fraction (*a*) by repeated freezing and thawing, (*b*) in hypo-osmotic sucrose at 0°, (*c*) at different pH values at 0°, (*d*) at different temperatures. The acetylcholine released is expressed as a percentage of the sum of released and bound acetylcholine remaining at the end of the experiment. (*a*), Rabbit; (*b–d*), guinea pig. Total acetylcholine of fractions (*a*) 2·8, (*b*) 3·0, (*c*) 3·6, (*d*) 2·8 μm-moles/ml.

Table 3. *Effect of various substances on release of acetylcholine from P_2 fraction of guinea-pig brain in sucrose containing eserine*

All experiments were carried out at 0° unless otherwise stated. Total acetylcholine of fractions (μm-moles/ml.): (*a*) 4·8, (*b*) 4·4, (*c*) 1·6, (*d*) 5·6, (*e*) 4·0, (*f*) 3·2; (*a*) eserine-free P_2, (*c*) eserine-free homogenate.

Agent	Concn. (mM unless otherwise stated)	Time of action (min.)	Percentage release of acetylcholine
Inorganic ions			
KCl	20	15	3(*a*)
	50		8(*c*)
$CaCl_2$	5		0(*b*)
	10		13(*b*)
	20		7(*b*)
	50		13(*b*), 8(*c*)
	100		7(*b*)
	200		7(*b*)
$MgCl_2$	20		3(*a*)
	50		2(*a*)
Organic solvents			
Chloroform, Ether, Light petroleum (b.p. 40–60°)	sat. soln.	10	100(*d*), 100(*d*), 11(*f*)
Biochemical preparation			
Cobra venom (tested at 22°)	30 μg./ml.	10, 60	50(*d*)
Drugs			
5-HT	0·05	5, 30	0(*e*)
Reserpine	10 μg./ml.	30, 230	0(*e*)
DDT*	0·1	30, 90	0(*e*)

* 1:1:1-Trichloro-2:2-di-(*p*-chlorophenyl)ethane.

Temperature. The effect of temperature on the release of acetylcholine is shown in Fig. 6*d*. It will be seen that acetylcholine is almost completely released by incubating the particles at 37° for 1 hr. At 22°, only about 10% of the acetylcholine was released in this time. At 0° release is extremely slow, having reached only 54% in 3 days. The bound particulate acetylcholine still present at this time was all in the 'stable' form, as was shown by its release by ether or acid treatment but not by exposure to 0·032M-sucrose. Thus 'labile' acetylcholine seems to be lost preferentially.

Inorganic ions, organic substances and enzymes. The result of these experiments are summarized in Table 3. K^+, Mg^{2+} and Ca^{2+} ions had little effect, though their presence caused the particles to clump and sediment more readily on centrifuging than did the control samples. Organic solvents (chloroform, ether) liberated bound acetylcholine completely. Cobra venom, in the highest concentration tested, liberated only about 50% of the particulate acetylcholine. Presumably the lecithinase in the venom damages the particles sufficiently to allow the labile fraction of particulate acetylcholine to diffuse out, but does not affect the stable fraction. The stable fraction could be released by treating the cobra-venom-treated particles with ether.

Effect of cholinesterase. As already mentioned, brain cholinesterase does not destroy particulate acetylcholine (as shown by the survival of this in preparations made in eserine-free sucrose) although it does destroy free acetylcholine. The effect of cholinesterase on particulate acetylcholine was, therefore, studied further with a highly purified preparation of bovine-red-cell cholinesterase. The results obtained are illustrated in Table 4. It will

Table 4. *Action of cholinesterase on particulate acetylcholine*

The P_2 fraction was prepared from an eserine-free sucrose homogenate of guinea-pig brain. Water-treated particles were obtained by centrifuging a sample of P_2 and resuspending the precipitate in an equal volume of water. Acetylcholine and enzyme (purified bovine-red-cell cholinesterase, Stearns Winthrop Inc.; activity 30 u./mg.) were added in 0·32M-sucrose, with extra 0·32M-sucrose where necessary to give a total volume of 1·2 ml. in each tube. The tubes were kept for 10 min. at room temperature and the reaction was stopped by the addition of eserine (5 μg. in 0·05 ml.). The tubes were brought to pH 4 and heated for 10 min. at 100° to release bound acetylcholine before assay.

Tube	Additions	Total acetylcholine remaining (μm-moles)
1	Acetylcholine (10 μm-moles)	10
2	Acetylcholine + enzyme (0·5 mg.)	0
3	Particles (1 ml. of P_2)	3·1
4	Particles + enzyme	3·2
5	Particles + acetylcholine + enzyme	4·0
6	Water-treated particles (1 ml.) + enzyme	1·2
7	Water-treated particles + acetylcholine + enzyme	1·2

be seen that added enzyme was unable to destroy particulate acetylcholine (compare tubes 3 and 4), although the enzyme destroyed three times as much free acetylcholine (compare tubes 1 and 2) in the same time. That this failure was not due to destruction or inhibition of the enzyme by the particles is shown in tube 5, in which the enzyme, acting on a mixture of the particles and added acetylcholine, destroyed an amount almost equal to that of free acetylcholine added. When particulate acetylcholine was partially released by treating the particles with water, the released acetylcholine was destroyed (tube 6), the amount of acetylcholine remaining corresponding to the stable fraction observed in experiments of the type illustrated by Fig. 6*b*. The survival of this fraction of acetylcholine was not due to inhibition of the added cholinesterase, since added acetylcholine was completely destroyed in the presence of the water-treated particles (tube 7). The change in the particles induced by water treatment is thus evidently not sufficiently drastic to permit access of cholinesterase to the stable fraction.

The amount of acetylcholine remaining in tube 5 was greater than in the corresponding control tube (4), although the concentration of enzyme should have been sufficient to destroy all the added acetylcholine. The particles appeared to have 'protected' about 8% of the added acetylcholine from the cholinesterase. Further investigation showed that this was due to an uptake of acetylcholine from the medium by the particles. It was not due to synthesis of acetylcholine by the particles from the products of the enzymic hydrolysis, since particles were unable to increase their acetylcholine content at the expense of an equivalent amount of choline and acetic acid added to the medium. Aged or water-treated particles or particles, acidified to pH 4·0 and then neutralized, were unable to take up acetylcholine from the medium. This suggests that the process is an active one and dependent on an intact membrane.

Inability of subcellular particles from liver to bind acetylcholine. The possibility was considered that the binding of acetylcholine by brain particles was an artifact. Accordingly rat liver, a tissue with a negligible acetylcholine content, was homogenized in 0·32 M-sucrose in the presence and absence of 50 μM-eserine and acetylcholine (10 μm-moles/ml.), and then separated into the various subcellular fractions as for brain. If eserine was present, all the acetylcholine was recovered in the supernatant fraction; if eserine was absent, all the acetylcholine was destroyed, presumably by the liver cholinesterase. When acetylcholine (10 μm-moles/ml.) was added to an eserine-free P_2 fraction from rat liver, there was a steady fall in free acetylcholine, unaccompanied by any uptake of acetylcholine by the particles. While it is obviously impossible to reproduce the conditions prevailing in brain with another tissue, these experiments certainly do not support the idea that the binding of acetylcholine by brain particles is an artifact.

Model experiments with an ion-exchange resin. Since the critical range of pH in which the stable fraction of bound acetylcholine begins to be displaced from the particles is between 4·0 and 4·5 (Fig. 5*c*), it was considered that the main bond between stably bound acetylcholine and the particle matrix might be an ionic one between the quaternary nitrogen of acetylcholine and a carboxylic acid group. Choline esters are known to be adsorbed by carboxylic acid ion-exchange resins (Gardiner & Whittaker, 1954; Keyl, Michaelson & Whittaker, 1957), so it was decided to compare the properties of bound acetylcholine with those of suspensions of resin containing adsorbed choline esters of varying affinity. Three pharmacologically active esters, acetyl-, *iso*valeryl- and urocanylcholine, of increasing affinity, were adsorbed on samples of the carboxylic acid resin XE-97 (Rohm & Haas Co., Philadelphia, Pa., U.S.A.) in column form. The resin was well washed and suspended in water. The supernatants from these suspensions were tested on the frog rectus and remained free of ester. When the suspensions themselves were tested, those containing acetylcholine were as active as would have been expected if all the acetylcholine had been displaced from the resin by the cations of the frog Ringer solution, and the ability of frog Ringer solution to displace the ester was confirmed. By contrast, resin suspensions containing the more strongly adsorbed *iso*valeryl- and urocanyl-choline were inactive. However, heating these suspensions at 100° for 10 min. at pH 4·0 displaced the esters and the supernatants from resins so treated were fully active. It thus seems likely that ionic forces alone would be unable to account for the stability of stable bound acetylcholine, but they might nevertheless provide the main component of the binding forces. The further study of these binding forces has been handicapped by the failure so far to discover ways of reversibly depleting the particles of acetylcholine.

Release of 5-hydroxytryptamine. Investigation of the release of 5-HT from particulate fractions was complicated by (*a*) the spontaneous release during assay, referred to in a previous section, (*b*) destruction of released 5-HT. Provided that the monoamine oxidase inhibitor 1-*iso*nicotinoyl-2-*iso*propylhydrazine phosphate (iproniazid) was added beforehand in a final concentration of mM, the pattern of release of 5-HT resembled in general that of acetylcholine in the presence of eserine, although iproniazid was not as effective as a stabilizing agent for 5-HT as eserine was for

acetylcholine. Thus ether treatment resulted in total release of particle-bound 5-HT, while hypo-osmotic dilution (resuspension of particles from the P_2 fraction in 0·032 M-sucrose) gave evidence of a labile fraction of bound 5-HT, a maximum of 40–50 % of the total 5-HT being liberated under these conditions after 45 min. In the absence of iproniazid, liberated 5-HT was rapidly destroyed, as was acetylcholine in the absence of eserine.

The behaviour of 5-HT, however, did not exactly parallel that of acetylcholine. Even in the absence of iproniazid, there was free 5-HT present in the supernatant fraction of sucrose homogenates and added 5-HT was stable in contact with untreated particles. Nevertheless, disruptive treatment led to destruction of added 5-HT as well as of released endogenous 5-HT unless iproniazid were added beforehand. It was therefore concluded that, unlike cholinesterase, the iproniazid-sensitive factor (presumably monoamine oxidase) responsible for the destruction of 5-HT is present in an inactive form in untreated particles and is released under the same conditions as is 5-HT. Evidence that monoamine oxidase is not freely accessible *in vivo* has been presented by Udenfriend, Weissbach & Bogdanski (1957). Normal methods of determining monoamine oxidase activity *in vitro* would be expected to be highly disruptive to intracellular particles and would fail to reveal the existence of an intracellular permeability barrier to substrates and inhibitors.

DISCUSSION

The resolution of homogenates into morphologically identifiable constituents is much more difficult with brain than with liver, the tissue most investigated from this point of view. The so-called mitochondrial fraction is much more complex; nevertheless, it has been successfully separated into three well-defined subfractions on the basis of density differences. The densest of these has been shown to consist mainly of mitochondria and might be a convenient starting point for a pure preparation of these organelles for metabolic studies. The lightest consists of relatively large, structureless particles which have not so far been identified. The particles of intermediate density have been distinguished from mitochondria on both morphological and biochemical evidence and contain a significant proportion of the bound acetylcholine and 5-HT of the brain as well as much of the acid phosphatase. The only well-defined structures known to be present in the central nervous system with a comparable size range are the synaptic vesicles. Even if these are in reality tubules rather than vesicles, as suggested by some investigators (Gray, 1959), it is conceivable that they might break up on homogenization to give particles of the size range observed, just as the smaller endoplasmic reticulum is believed to give rise to microsomes.

The presence in this subfraction of two pharmacologically active components with different distributions in whole brain suggest that it consists of particles of more than one type; however, the conditions under which acetylcholine and 5-HT are released are sufficiently similar to suggest that the particles containing these two substances are closely related. Since material denser than 1·2 M-sucrose was not present in this preparation, the 5-HT particles in brain must differ from those isolated from intestinal mucosa by Baker (1958). Previous evidence on the localization of bound 5-HT in brain has been conflicting (Walaszek & Abood, 1957; Bogdanski, Weissbach & Udenfriend, 1956).

A study of the factors influencing the release of acetylcholine from the particles has not enabled the mechanism of acetylcholine release *in vivo* to be identified as yet. The particles appear to be stable at 0° to ions such as Ca^{2+}, Mg^{2+} and K^+, though the presence of these ions caused them to coacervate, so that it is unlikely that ionic shifts themselves are the determining factor. However, this study has confirmed earlier suggestions that about half of the bound acetylcholine is present in a highly labile form and behaves as though it were imprisoned in the free state inside a membrane which is readily damaged by osmotic dilution, freezing and thawing and other mildly disruptive procedures. The remaining portion of the particle-bound acetylcholine is more firmly attached and requires more drastic conditions for release—incubation at 37°, exposure to pH values of 4·0 or less or treatment with organic solvents. This implies a fairly strong chemical bond between acetylcholine and the particle matrix. The most obvious one would be an ionic bond between the quaternary nitrogen of acetylcholine and an acidic group, reinforced perhaps by other short-range intermolecular forces of the type that have been discussed in connexion with the binding of esters by cholinesterase (Adams & Whittaker, 1950) and other specific biological interactions. The model experiments with a carboxylic acid ion-exchange resin containing adsorbed choline esters gave limited support to this idea.

The concept of the acetylcholine-containing particle which emerges from this work is summarized in Fig. 7. The particle is envisaged as a simple vesicle, about 0·05 μ in diameter, containing some acetylcholine in solution and some bound ionically to carboxylic acid groups in the particle wall. This fraction of acetylcholine is shown within the particle, because it remains inaccessible to cholinesterase even when the permeability of the particle is increased by mild disruptive techniques.

The particle is also shown to contain choline acetylase (Hebb & Whittaker, 1958) but not acetocholinesterase, because recent work by Toschi (1959) and Aldridge & Johnson (1959) has shown that this component of the acetylcholine system is localized primarily in the microsomal fraction and that its presence in other fractions is due to microsomal contamination. Toschi has also found that, when the microsomal fraction is dissociated into an RNA-rich portion (consisting mainly of Palade granules) and an RNA-poor fraction (consisting mainly of structureless vesicles derived from the membranous component of the endoplasmic reticulum), the acetocholinesterase is concentrated in the latter portion. The dissociation between RNA and acetocholinesterase is also shown by the more diffuse distribution of the latter among the primary fractions of the sucrose homogenate (Aldridge & Johnson, 1959; V. P. Whittaker, unpublished observations), suggesting that the acetocholinesterase may be bound to RNA-poor portions of the endoplasmic reticulum. Since in current cytochemical thinking no clear distinction is made between the limiting membrane of the cell and the endoplasmic reticulum, these RNA-poor portions of the latter might correspond to regions of the postsynaptic neurone nearest to the presynaptic nerve terminals. The suggested relationship of the two types of particles to each other and to the morphology of the synapse is indicated in Fig. 7.

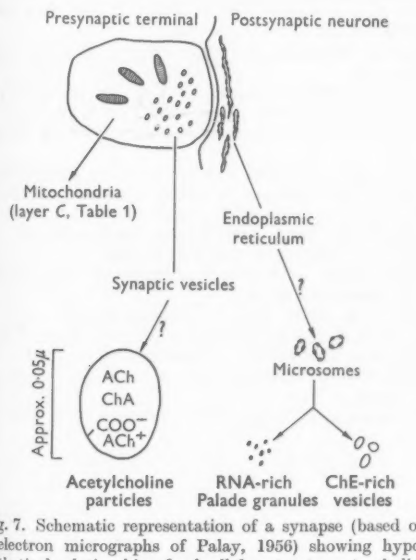


Fig. 7. Schematic representation of a synapse (based on electron micrographs of Palay, 1956) showing hypothetical relationship of subcellular structures to cholinesterase-rich vesicles (Toschi, 1959) and acetylcholine particles (this paper). Abbreviations: ChE, acetocholinesterase; ACh, acetylcholine; ChA, choline acetylase.

The behaviour of bound acetylcholine as revealed by the experiments carried out on the P_2 fraction and the acetylcholine-rich fraction derived from it is in general consistent with most previous work on the subject, though much of this was done with homogenates in water or sodium chloride solution which must have contained acetylcholine particles in a more or less damaged state. Thus Cavanaugh & Tobias (1949) showed that bound acetylcholine was liberated from water homogenates of brain on incubation, and Brodkin & Elliott (1953) distinguished between two forms of bound acetylcholine in saline homogenates. The release of bound acetylcholine by ether parallels the activation of particulate choline acetylase by ether (Hebb & Smallman, 1956). The increased synthesis of acetylcholine observed in chloroform, or ether-treated brain minces (Stedman & Stedman, 1937, 1939) or venom-treated brain slices (Braganca & Quastel, 1952) is consistent with the release of acetylcholine from chloroform-, ether- or venom-treated particles (Table 3), and suggests that the rate-limiting factor in acetylcholine synthesis in inactive neurones may be the accumulation of intraparticulate acetylcholine.

So far as they show that procedures which drastically affect the integrity of the particles release all the bound acetylcholine, the present findings are not consistent with reports of an ethanol-soluble form of bound acetylcholine in brain (Abdon & Hammarskjöld, 1944; Loewi & Hellauer, 1938). An attempt to repeat these findings was unsuccessful; the acetylcholine extracted by ethanol from brain under conditions approximating to those described by Abdon & Hammarskjöld was all in the free form.

Although the two forms of particle-bound acetylcholine have been represented in Fig. 7 as occurring in the same particle, there is at present no conclusive evidence that this is so. There may be two kinds of particle or a spectrum of particles. However, the release of approximately 50 % of the bound acetylcholine by such diverse procedures as treatment with cobra venom, freezing and thawing, shaking with Ballotini beads and hypotonic dilution is difficult to reconcile with the second of these alternatives.

SUMMARY

1. The crude mitochondrial fraction of sucrose homogenates of rabbit and guinea-pig brain was resolved into three main fractions by equilibrium centrifuging in a density gradient. These were: *A*, particles less dense than 0·8 M-sucrose, diameter 2–5 μ; *B*, particles denser than 0·8 M-sucrose and lighter than 1·2 M-sucrose, diameter 0·02–0·3 μ

(median, 0·065 μ); *C*, particles denser than 1·2 M-sucrose, diameter 0·02–1·0 μ (median 0·25 μ).

2. The particles of fraction *C* were identified on morphological and biochemical evidence as mitochondria.

3. Most of the particle-bound acetylcholine and 5-hydroxytryptamine was localized in the *B* layer. Over 60 % of the particles of this fraction had the size range (0·02–0·08 μ) reported for synaptic vesicles, and appeared as simple vesicles in electron micrographs.

4. The distribution of two lysosome enzymes was also studied. One of these, acid phosphatase, was mainly in the *B* layer, the other, β-glucuronidase, mainly in the *C* layer. It would seem that the lysosome hypothesis requires modification when applied to brain.

5. A study was made of the factors determining the release of acetylcholine and 5-hydroxytryptamine. Further evidence was obtained for two forms of bound acetylcholine—a labile fraction released by mildly disruptive techniques which probably alter the permeability of the particles and permit the outward diffusion of free acetylcholine contained within them, and a more stable fraction which is held by chemical forces, including ionic links, to the particle matrix.

I am most grateful to Dr K. M. Smith and Mr G. J. Hills for the electron micrographs, to Dr W. N. Aldridge for access to unpublished work, and to Dr J. H. Gaddum for demonstrating to me the applicability of the lognormal distribution to the study of particle sizes and for the calculations embodied in Fig. 4. I am also greatly indebted to Miss J. M. Gilson and Mr G. Dowe for their skilful assistance.

REFERENCES

Abdon, N.-O. & Hammarskjöld, S. O. (1944). *Acta physiol. scand.* **8**, 75.
Adams, D. H. & Whittaker, V. P. (1950). *Biochim. biophys. Acta*, **4**, 543.
Aldridge, W. N. & Johnson, A. (1959). *Biochem. J.* (in the Press).
Baker, R. V. (1958). *J. Physiol.* **143**, 80*P*.
Beaufay, H., Berleur, A. M. & Doyen, A. (1957). *Biochem. J.* **66**, 32P.
Berthet, J. & de Duve, C. (1951). *Biochem. J.* **50**, 174.
Bodian, D. (1942). *Physiol. Rev.* **22**, 140.
Bogdanski, D. F., Weissbach, H. & Udenfriend, S. (1956). *J. Neurochem.* **1**, 272.
Braganca, B. M. & Quastel, J. H. (1952). *Nature, Lond.*, **169**, 695.
Brodkin, E. & Elliott, K. A. C. (1953). *Amer. J. Physiol.* **173**, 437.
Cavanaugh, D. J. & Tobias, J. M. (1949). *Fed. Proc.* **8**, 22.
Chang, C. H. & Gaddum, J. H. (1933). *J. Physiol.* **76**, 255.
Cohn, P. & Richter, D. (1956). *J. Neurochem.* **1**, 166.
Correale, P. (1957). *J. Neurochem.* **1**, 22.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
de Robertis, E. D. P. & Bennett, H. S. (1955). *J. biophys. biochem. Cytol.* **1**, 47.
de Robertis, E. & Franchi, C. M. (1956). *J. biophys. biochem. Cytol.* **2**, 307.
del Castillo, J. & Katz, B. (1956). *Progr. Biophys.* **6**, 121.
Eccles, J. C. (1957). *The Physiology of Nerve Cells*. Baltimore: The Johns Hopkins Press.
Feldberg, W. (1945). *Physiol. Rev.* **25**, 596.
Gardiner, J. E. & Whittaker, V. P. (1954). *Biochem. J.* **58**, 24.
Gray, E. G. (1959). *J. Physiol.* **145**, 25*P*.
Hebb, C. O. (1957). *Physiol. Rev.* **37**, 196.
Hebb, C. O. & Smallman, B. N. (1956). *J. Physiol.* **134**, 385.
Hebb, C. O. & Whittaker, V. P. (1958). *J. Physiol.* **142**, 187.
Hogeboom, G. H., Claude, A. & Hotchkiss, R. D. (1946). *J. biol. Chem.* **165**, 615.
Kerr, L. M. H. & Levvy, G. A. (1951). *Biochem. J.* **48**, 209.
Keyl, M. J., Michaelson, I. A. & Whittaker, V. P. (1957). *J. Physiol.* **139**, 434.
Kuff, E. L. & Schneider, W. C. (1954). *J. biol. Chem.* **206**, 677.
Loewi, O. & Hellauer, H. (1938). *Pflüg. Arch. ges. Physiol.* **240**, 449.
Luft, J. H. (1956). *J. biophys. biochem. Cytol.* **2**, Suppl. 229.
Novikoff, A. B. (1957). *Symp. Soc. exp. Biol.* **10**, 92.
Palay, S. L. (1956). *J. biophys. biochem. Cytol.* **2**, Suppl. 193.
Robertson, J. D. (1956). *J. biophys. biochem. Cytol.* **2**, 381.
Stedman, E. & Stedman, E. (1937). *Biochem. J.* **31**, 817.
Stedman, E. & Stedman, E. (1939). *Biochem. J.* **33**, 811.
Stone, W. E. (1955). *Arch. Biochem. Biophys.* **59**, 181.
Toschi, G. (1959). *Exp. Cell. Res.* **16**, 232.
Udenfriend, S., Weissbach, H. & Bogdanski, D. F. (1957). *J. Pharmacol.* **120**, 255.
Vane, J. R. (1957). *Brit. J. Pharmacol.* **12**, 344.
Walaszek, E. J. & Abood, L. G. (1957). *Fed. Proc.* **16**, 133.
Whittaker, V. P. (1958*a*). *Biochem. J.* **68**, 21P.
Whittaker, V. P. (1958*b*). *Abstr. 4th Int. Congr. Biochem., Vienna*, p. 74.
Whittaker, V. P. (1959). *Biochem. Pharmacol.* **1**, 351.

The Reaction of Tetrahydropteroylglutamic Acid and Related Hydropteridines with Formaldehyde

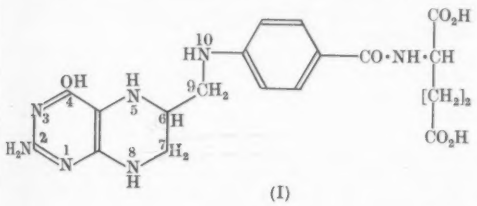
By R. L. BLAKLEY
Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T.

(*Received* 17 *December* 1958)

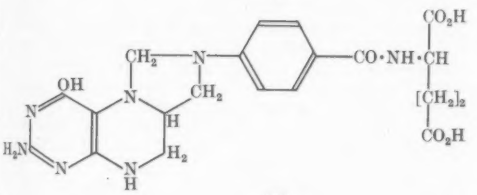
Since the discovery that tetrahydropteroylglutamic acid (I) stimulates the synthesis of serine from glycine and formaldehyde or formate in liver-enzyme preparations (Blakley, 1954*a*, *b*; Kisliuk & Sakami, 1954), there has been considerable speculation on the chemical mechanism by which this acid activates formaldehyde in this reaction. On the basis of known reactions of formaldehyde with 1:2-diamines it seemed likely that formaldehyde would condense with two amine groups to form an imidazolidine structure, N^5N^{10}-methylenetetrahydropteroylglutamate (II) (Blakley, 1954*b*), although an alternative is the formation of N^5-hydroxymethyltetrahydropteroylglutamate (III), as suggested by Kisliuk & Sakami (1954).

Tetrahydropteroylglutamic acid is extremely sensitive to oxidation and in air rapidly breaks down to xanthopterin, pteroylglutamic acid and other products (Blakley, 1957*a*). On the other hand, leucovorin, the N^5-formyl derivative of the acid, is relatively stable to oxidation, the formyl group protecting the hydrogenated pyrazine ring. It might be expected therefore that N^5N^{10}-methylenetetrahydropteroylglutamate would also be resistant to oxidation. But formaldehyde in low concentrations did not protect tetrahydropteroylglutamate from oxidation to any significant extent (Blakley, 1957*a*); it was therefore concluded that under these conditions the N^5N^{10}-methylene derivative is not formed in appreciable amounts.

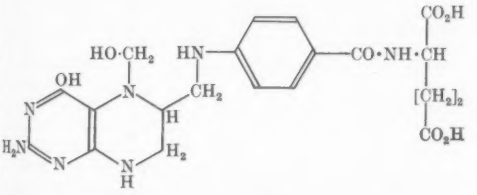
On the other hand, Kisliuk (1957), in a study of the non-enzymic binding of formaldehyde by tetrahydropteroylglutamic acid and other hydropteridines, concluded that a single molecule of formaldehyde must combine with both N-5 and N-10 of the tetrahydropteroylglutamic acid molecule forming the bridged structure (II). According to his results N^{10}-methyl- and N^5-formyl-tetrahydropteroylglutamic acid and 2-amino-4-hydroxy-6-methyltetrahydropteridine were unable to bind significant amounts of formaldehyde; it was concluded that when combination of a single molecule of formaldehyde with two amine groups of tetrahydropteroylglutamate occurred a stable product was formed. As preliminary results from this Laboratory on the combination of $H^{14}CHO$ with hydropteridines (Blakley, 1957*c*) had indicated a combination of formaldehyde both with N^{10}-formyltetrahydropteroylglutamic acid and with 2-amino-4-hydroxy-6-methyltetrahydropteridine, further study of the combination of formaldehyde with these and other hydropteridines seemed desirable. Moreover, since all the tetrahydropteridines previously examined for reaction with formaldehyde are attacked extremely rapidly by dissolved oxygen, it was of interest to investigate at least one stable tetrahydropteridine and for this purpose 4-hydroxy-6-methyltetrahydropteridine has been synthesized. The results of these studies, here presented, indicate that formaldehyde combines non-enzymically with all hydropteridines, but that the extent of combination with tetrahydropteroylglutamic acid is greater than with any other hydropteridine studied.



(I)



(II)



(III)

Osborne, Vercamer, Talbert & Huennekens (1957) have reported the isolation from acetone-dried pigeon liver of an enzyme which catalyses the combination of formaldehyde and tetrahydropteroylglutamic acid as shown by spectrophotometric studies. Partially purified serine transhydroxymethylase preparations used in this Laboratory have been examined for such an enzyme and attention has been directed to the question whether non-enzymic combination of formaldehyde and tetrahydropteroylglutamic acid is sufficiently rapid to account for observed rates of enzymic serine synthesis. A brief summary of these results has been published elsewhere (Blakley, 1958).

EXPERIMENTAL

Materials

Pteridines. Commercial samples of folic acid were purified as previously described (Blakley, 1957*a*). A specimen gave the following analysis (Found: C, 48·3; H, 4·81; N, 20·6. $C_{19}H_{19}O_6N_7,2H_2O$ requires C, 47·8; H, 4·86; N, 20·6%). Samples of pteroic acid, 2-amino-4-hydroxy-6-methylpteridine, leucovorin and N^{10}-methylpteroylglutamic acid were generously donated by Dr H. P. Broquist of Lederle Laboratories, Pearl River, New York. The following compounds were synthesized by Dr D. J. Brown of the Department of Medical Chemistry of this University: 4:5-diaminopyrimidine (Brown, 1952); 2-deaminopteroylglutamic acid (Brown, 1953); 4-hydroxy-6-methylpteridine (Albert, Brown & Cheeseman, 1952); N^8-benzyl-2-chloro-4-methyltetrahydropteridine (Brook & Ramage, 1955); tetrahydroquinoxaline (Merz & Ris, 1887).

N^{10}-*Formylpteroylglutamic acid.* Since published methods for the synthesis of N^{10}-formylpteroylglutamic acid gave products which were often found by paper electrophoresis to be grossly contaminated, the following procedure was adopted. Pteroylglutamic acid (approximately 0·5 g. of purified material) was dissolved in 98% formic acid (20 ml.) without heating and allowed to stand for 2 days at 20°. The formic acid was removed by freeze-drying and the product dissolved in hot water (200 ml.) and filtered. The filtrate, together with the material which precipitated from it on cooling, was immediately freeze-dried, to yield a fluffy cream residue. When the product was subjected to chromatography and electrophoresis on paper, examination of the papers in ultraviolet light revealed the presence of one major component with a blue fluorescence, two other fluorescing components which were present only in small amounts and a small amount of folic acid. The impurities were removed by chromatography on Whatman no. 3 sheets (20 mg. of pteridine/sheet) with 0·1 M-ammonium formate as solvent. The major fluorescing band was eluted with 0·1 M-ammonium formate buffer (pH 9) and the eluate freeze-dried. The residue was dissolved in a small volume of hot water and N^{10}-formylpteroylglutamic acid precipitated by adjusting to pH 4 with formic acid and allowing the flask containing the solution to stand in a large beaker of hot water in the dark until the whole system had cooled to room temperature. The precipitate was recovered by centrifuging, washed with cold water and finally dissolved in hot water (20 ml.) and freeze-dried. Residual ammonium formate was sublimed off at 100° under vacuum (5×10^{-2} mm. Hg). The product, dissolved in 0·01 N-NaOH to give a concentration of 10·8 μg./ml., had absorption maxima at 256 and 365 mμ and minima at 225 and 305 mμ. The molecular extinction at 256 mμ was 46 000, which is considerably higher than that for material prepared by other methods (cf. Pohland, Flynn, Jones & Shive, 1951). After standing overnight in N-NaOH at room temperature, the compound was quantitatively converted into pteroylglutamic acid as shown by spectra and paper chromatography.

Tetrahydropteroylglutamic acid (tetrahydroPGA), dihydropteroylglutamic acid (dihydroPGA) and tetrahydro-N^{10}-formylpteroylglutamic acid (N^{10}-formyltetrahydroPGA) were prepared by catalytic hydrogenation as previously described (Blakley, 1957*a*). DihydroPGA was also prepared by the method of Futterman (1957). If an aqueous solution of tetrahydroPGA is stored under H_2 in the presence of platinum catalyst (20 mg. for 4 ml. of 0·01 M-tetrahydroPGA), further uptake of H_2 occurs until 6 molecular proportions of H_2 have been consumed. The rate at which this occurs varies with the state of aggregation of the catalyst but is reduced to a very low level by storing solutions under a low pressure of H_2 (20 mm. Hg) at 0°; this procedure was therefore adopted for keeping tetrahydroPGA solutions for periods up to a week. Little liberation of aromatic amine occurred (0·08 mole/mole of pteridine) during hydrogenation beyond the tetrahydro stage. In the enzymic synthesis of serine this material had low coenzyme activity, the Michaelis constant being 1×10^{-3} M (cf. tetrahydroPGA below).

Dihydropteroic acid and 2-amino-4-hydroxy-6-methyltetrahydropteridine were prepared by catalytic hydrogenation in aqueous 0·01 N-NaOH.

4-*Hydroxy-6-methyltetrahydropteridine.* To a suspension of 4-hydroxy-6-methylpteridine (1·6 g.) in water (50 ml.) at room temperature solid sodium borohydride (0·57 g.) was added slowly, with stirring, over a period of 15 min. The solution was allowed to stand for a further 15 min. at room temperature before being freeze-dried. The residue was extracted with several portions of hot propan-1-ol (total 70 ml.), insoluble material being removed by centrifuging after each extraction. The combined extracts were concentrated by heating under a stream of N_2 until the volume was about 10 ml. Sufficient water was added to the hot mixture to give a clear solution, which was then allowed to stand overnight at 0°. The crystals which formed were filtered off in the cold and washed with cold propan-1-ol. After drying *in vacuo* over P_2O_5 the yield of the cream-coloured crystals was 0·65 g. Further crops of crystals were obtained by concentrating the mother liquor. A portion of this material was twice recrystallized from propan-1-ol–water (1:1). After drying for 30 min. at 140° over P_2O_5 (5×10^{-2} mm. Hg) the following analysis was obtained (Found: C, 50·2; H, 6·01; N, 33·3. $C_7H_{10}ON_4$ requires C, 50·6; H, 6·07; N, 33·7%).

When the recrystallized material was subjected to chromatography in several solvent systems and the chromatograms were inspected in ultraviolet light, only one spot could be detected. This had a faint-blue fluorescence. Titration of a 5 mM-solution with 0·1 N-KOH revealed an acidic group with pK 10·20 $\pm$ 0·03 and indicated the presence of only one compound. The material melted at 112–116° (uncorr.).

N^5-Formyl-4-hydroxy-6-methyltetrahydropteridine. 0·65 g. of 4-hydroxy-6-methyltetrahydropteridine, once-crystallized, was dissolved in 98% formic acid (8 ml.); acetic anhydride (2 ml.) was added and the solution kept overnight at room temperature. The solution was evaporated to dryness under a low pressure of N_2, 5 ml. of ethanol added and evaporation to dryness repeated. The residue was extracted with boiling ethanol (15 ml.) and then dissolved in the minimum volume of hot water. The ethanolic and aqueous solutions were mixed and allowed to stand overnight at 0°. The white crystals which formed were filtered off and dried *in vacuo* over P_2O_5 at room temperature. Yield was 0·435 g. The material was recrystallized from ethanol–water and dried for 1 hr. at 140° over P_2O_5 (5×10^{-2} mm. Hg) and gave the following analysis (Found: C, 49·8; H, 5·11; N, 28·4. $C_8H_{10}O_2N_4$ requires C, 49·5; H, 5·19; N, 28·8%). In a melting-point tube the crystals softened at 251° and melted with decomposition at 257–259° (uncorr.).

Serine transhydroxymethylase. This was prepared by the method previously described (Blakley, 1955), except that advantage was taken of the stabilizing effect of ethylenediaminetetra-acetic acid (EDTA) on the enzyme to achieve a useful fractionation with acetone. By this means a preparation was obtained with activity four to five times that obtained previously.

The rabbit-liver powder (20 g.), prepared as before, was extracted for 1 min. in a Waring Blendor with 300 ml. of cold 0·01 M-potassium phosphate buffer (pH 7·5) containing mM-EDTA. The mixture was centrifuged and the supernatant heated at 53° as previously described. The supernatant from this treatment was brought to pH 6·4 by the addition of 0·03 vol. of M-potassium phosphate, pH 6·3, followed by 0·0075 vol. of M-KH_2PO_4, and then fractionated with acetone (purified by boiling with potassium permanganate and distilling over dry potassium carbonate), the temperature of the solution being progressively lowered from 0° to −4° as the acetone concentration was increased to 29% (v/v). The material which precipitated was removed by centrifuging at −5° and discarded. Acetone was added to the supernatant to bring the acetone concentration to 40% (v/v), the temperature being simultaneously lowered from −4° to −7°. The precipitate was removed by centrifuging and redissolved in 10 ml. of ice-cold 0·01 M-potassium phosphate buffer (pH 7·3) containing mM-EDTA. To this solution was added 1·0 vol. of an ice-cold solution of saturated ammonium sulphate containing mM-EDTA and adjusted with aqueous NH_3 solution (sp.gr. 0·880) so that, on dilution with an equal volume of water, it was pH 8. The precipitate was removed by centrifuging at 0° and discarded. More saturated ammonium sulphate solution was added to the supernatant to bring the final concentration of ammonium sulphate to 66% saturated. The precipitate was removed by centrifuging and redissolved in 10 ml. of cold 0·01 M-potassium phosphate buffer (pH 7·3) containing mM-EDTA. The solution was then fractionated with cold, saturated ammonium sulphate containing mM-EDTA and 50 ml. of M-potassium phosphate buffer (pH 7·3)/l. On dilution with an equal volume of water it was pH 6·4. Sufficient of the ammonium sulphate solution was added to bring the concentration of ammonium sulphate to 45% saturated. The precipitate was removed by centrifuging at 0° and discarded. More of the ammonium sulphate solution was added to the supernatant to bring the concentration of ammonium sulphate in the solution to 60% saturated. The precipitate was collected by centrifuging at 0°, and dissolved in 0·01 M-potassium phosphate buffer (pH 7·3, containing mM-EDTA) and the solution dialysed overnight against the same buffer.

Methods

Determination of enzyme activity. This was carried out manometrically as previously described (Blakley, 1955).

Determination of the binding of formaldehyde by hydropteridines. The reaction mixture contained 0·1 M-potassium phosphate buffer (pH 7·2), pteridine and formaldehyde in a total volume of 2·0 ml. After reaction at 20° (for 5 min. unless otherwise stated) the mixture was pipetted on to a column (3 cm. × 1 cm.) of Nuchar C (granular grade, West Virginia Pulp and Paper Co., New York). Before use the charcoal was boiled with 10% HCl, washed free of acid, dried at 95° and sieved to remove material finer than 40 mesh.

The reaction tube was washed with 2 ml. of water and the washings were pipetted on to the charcoal column after the reaction mixture had all entered the column. This procedure was repeated and the column was then washed with water until 20 ml. of effluent had been collected. The unbound formaldehyde which appeared in the effluent was determined in a sample by the method of Nash (1953). Bound formaldehyde was calculated by difference. Blank determinations were carried out in the same way except that no formaldehyde was added to the reaction mixture. Recovery of known amounts of formaldehyde in the absence of hydropteridine was also checked regularly.

Measurement of reaction rates. The reaction mixture containing formaldehyde (4 μmoles) and buffer was added to each of a series of conical flasks. At zero time the hydropteridine solution was added. After reaction at 20° for the stated periods 400 mg. of acid-washed Nuchar C was added to each and thoroughly mixed with the solution. Each mixture was poured into a chromatography column containing a cotton-wool plug, the effluent collected and again passed through the column. The charcoal was then washed with water until a total of 20 ml. of effluent had been collected. The formaldehyde contained in the filtered effluent was then determined by the method of Nash (1953).

Buffers. Potassium phosphate and sodium acetate buffers of the stated (anion) concentrations and pH were prepared from the data of Green (1933). Borate was used as the sodium salt.

RESULTS

Determinations of the amount of formaldehyde bound by reaction with various hydropteridines yielded the results presented in Table 1. Tetrahydroquinoxaline was also tested because of its close structural relationship to tetrahydropteridine, which is difficult to prepare.

Measurement of formaldehyde-binding by tetrahydroPGA and its derivatives was complicated by the fact that solutions of these compounds produce a colour in the formaldehyde estimation. Thus with a freshly prepared solution of tetrahydroPGA the colour produced was equivalent to almost 2 moles of formaldehyde for each mole of tetrahydroPGA, making it impossible to determine formaldehyde

directly in the presence of tetrahydroPGA. No explanation can be offered for the discrepancy between this result and the claim made by others that formaldehyde bound by tetrahydroPGA may be estimated directly by this colorimetric method (Osborne *et al.* 1957). If the solution of tetrahydroPGA was first run through a 3 cm. column of charcoal, as in the actual procedure adopted, 0·1–0·4 mole of 'formaldehyde' appeared in the effluent for each mole of tetrahydroPGA in the original solution. N^{10}-MethyltetrahydroPGA gave a particularly high blank value and only approximate results could be obtained with this substance. The 'formaldehyde blank' arising from hydropteridines has been subtracted from observed values in calculating free formaldehyde in solution. Although this relatively high blank value has limited the accuracy of the results for these compounds, it has not affected the validity of the conclusions. The blank value with 6-methylhydropteridines and tetrahydroquinoxaline was negligible.

Since reasonably reproducible results were obtained for the dissociation constants of the products formed by the reaction of the various hydropteridines with formaldehyde in widely varying proportions, it may be concluded that only minor disturbances of the base–formaldehyde equilibrium could have occurred during the washing of the column. Moreover, further prolonged washing of the column with water after 20 ml. of effluent had been collected removed a negligible amount of additional formaldehyde, even though a considerable proportion of the original formaldehyde was still bound on the column. This indicates that once the base–formaldehyde complexes were adsorbed on the charcoal they no longer dissociated to release formaldehyde.

The extent of the reaction of tetrahydroPGA with formaldehyde was unequalled by any other of the compounds tested, each mole of tetrahydroPGA binding 0·7–0·95 mole of formaldehyde. This is in agreement with the results obtained with $H^{14}CHO$ (Blakley, 1957*c*). Since in solution tetrahydroPGA reacts rapidly with dissolved oxygen, it seemed possible that stoicheiometric reaction between formaldehyde and tetrahydroPGA might be obtained if the latter were protected from oxygen. Accordingly the reaction was performed in the presence of 0·01 M-ascorbic acid, but no increase in the amount of bound formaldehyde was obtained. To check whether products of the oxidation of tetrahydroPGA had an appreciable capacity to find formaldehyde, the reaction of formaldehyde with L(+)-*p*-aminobenzoylglutamic acid and with

Table 1. *Binding of formaldehyde by hydropteridines and tetrahydroquinoxaline*

For method and conditions of reaction see text. Figures in parentheses are approximations.

Base	Base concn. (mM)	HCHO concn. (mM)	HCHO bound (% of total)	Dissociation constant
4-Hydroxy-6-methyltetrahydropteridine	50	5·0	61	0·031
	5	5·0	17	0·020
	50	2·5	60	0·032
	25	2·5	32	0·051
	25*	2·5	24	0·077
N^5-Formyl-4-hydroxy-6-methyltetrahydropteridine	50	5·0	10	(0·4)
2-Amino-4-hydroxy-6-methyltetrahydropteridine	10	2·5	20	0·038
	50	5·0	66	0·024
	20‡	5·0	25	0·056
8-Benzyl-2-chloro-4-methyltetrahydropteridine	33†	5·0	10	0·29
Tetrahydroquinoxaline	5*	2·5	32	0·009
	50	2·5	82	0·011
'Dihydropteroic acid'	10	5	92	0·00047
Dihydropteroylglutamic acid	5	5·0	27	0·0099
	5‡	5·0	25	0·011
Tetrahydropteroylglutamic acid	5	5·0	85	0·00013
	5‡	5·0	70	0·00064
N^{10}-Formyltetrahydropteroylglutamic acid	5‡	5·0	24	0·012
N^{10}-Methyltetrahydropteroylglutamic acid	22	2·2	(38)	(0·035)
N^5-Formyltetrahydropteroylglutamic acid (leucovorin)	17	2·5	4	(0·4)
2-Deaminotetrahydropteroylglutamic acid	5	5·0	18	0·019
	10	5·0	36	0·015

* Reaction was performed in ethanol–water (3:1) instead of in water.
† Reaction was performed in ethanol–water (19:1) instead of in water.
‡ Reaction mixture contained 0·01 M-ascorbic acid.

4:5-diaminopyrimidine was investigated. Both compounds reacted with formaldehyde, giving products with dissociation constants of 0·0036 and 0·064 M respectively. Consequently it is unlikely that the binding of formaldehyde by products of the degradation of tetrahydroPGA has caused a serious error in the figure for the amount of formaldehyde bound by the latter compound. The incompleteness of the reaction between formaldehyde and tetrahydroPGA is consistent with a reversible reaction, and the data give the approximate value of the dissociation constant, defined by

$$K = [\text{HCHO}]\,[\text{tetrahydroPGA}]/[\text{product}],$$

as 0·0004 (Table 1).

A group of compounds related to tetrahydroPGA were found to bind 0·04–0·46 mole of formaldehyde/mole of base, corresponding to dissociation constants of 0·017–0·0005 (Table 1). It may be noted that dihydroPGA, N^{10}-formyltetrahydroPGA and (as far as can be determined) N^{10}-methyltetrahydroPGA bind significant amounts of formaldehyde, whereas N^5-formyltetrahydroPGA (leucovorin) binds only very small amounts.

The very slight ability of leucovorin to bind formaldehyde indicates that N-10, N-8, N-3 and the 2-amino group of the tetrahydroPGA molecule have little reactivity towards formaldehyde. In N^{10}-formyltetrahydroPGA and N^{10}-methyltetrahydroPGA a methylene-bridge product similar to (II) is not possible, so that formaldehyde bound is presumably combined as an N-5-hydroxymethyl group. Because of difficulty in measuring the small hydrogen uptake by the sample of pteroic acid, the 'dihydropteroic acid' may have contained considerable amounts of tetrahydropteroic acid. This would be consistent with the large amount of formaldehyde bound by the hydropteroic acid.

Tetrahydroquinoxaline and 6-methyltetrahydropteridines combined 0·02–0·17 mole of formaldehyde/mole of base, corresponding to dissociation constants of 0·01–0·04, indicating that while the *p*-methylaminobenzoyl-L-glutamic acid side-chain considerably increases the affinity of hydropteridines for formaldehyde, appreciable reaction still occurs between formaldehyde and compounds lacking the long side-chain. In such cases also, N^5-hydroxymethyltetrahydropteridines are the most likely products.

N^5-Formyl-4-hydroxy-6-methyltetrahydropteridine, like N^5-formyltetrahydroPGA, bound negligible quantities of formaldehyde, again indicating that a free N-5-amine group is necessary for reaction with formaldehyde.

The fact that N^8-benzyl-2-chloro-4-methyltetrahydropteridine did not combine with formaldehyde might be due to the influence of the N-8-benzyl group on the reactivity of N-5. Further studies with related compounds are necessary, however, for an adequate understanding of the lack of reactivity of this compound.

Effect of pH *and formaldehyde concentration on formaldehyde-binding by tetrahydropteroylglutamic acid*

The amount of formaldehyde bound by tetrahydroPGA in the presence of various concentrations of formaldehyde is shown in Table 2. As the excess of formaldehyde present was increased the errors in the estimate of the amount bound became proportionately greater. It may be seen, however, that a fourfold increase in formaldehyde concentration did not significantly increase the amount of bound formaldehyde beyond 1 mole/mole of tetrahydroPGA.

When tetrahydroPGA (5 mM) was allowed to react with formaldehyde (10 mM) for 1 min. the amount of formaldehyde bound was close to one molecular proportion at all pH values tested between pH 3·8 and 13·0 (Table 2). Somewhat less formaldehyde was bound at pH 1. These results suggest that the N-5-amine group of tetrahydroPGA has a pK of about 1.

Rate of reaction between tetrahydropteroylglutamic acid and formaldehyde

Measurements at the lowest concentrations (0·2 mM) of tetrahydroPGA and formaldehyde at which analyses could be performed indicated that combination of these compounds was complete in about 10 min. (Fig. 1), and that 50% of the formaldehyde had reacted in the first minute. Reaction rates were too rapid to permit investigation of the effect of increased formaldehyde concentration. Since the reaction velocity will be

Table 2. *Effect of* pH *and formaldehyde concentration on the amount of formaldehyde bound by tetrahydropteroylglutamic acid*

TetrahydroPGA at a concentration of 5 mM was caused to react with formaldehyde at the concentrations indicated in a total volume of 2 ml. under standard conditions as described in the Methods section.

Formaldehyde concn. (mM)	Buffer	pH	Formaldehyde bound (μmoles)
5	0·1 M-Phosphate	7·4	9·1
10		7·4	10·8
20		7·4	11·8
10		7·4	9·8
10		6·3	8·3
10	0·1 M-Acetate	5·0	9·3
10	0·01 M-Acetate	3·8	8·8
10	0·005 M-Borate	9·2	8·9
10	0·1 N-NaOH	13	8·7
5	0·1 M-Phosphate	7·3	7·9
5	0·01 M-Acetate	3·8	6·1
5	0·1 N-HCl	1·0	5·3

proportional to formaldehyde concentration, it may be calculated that, with a formaldehyde concentration of 0·01 M as used in the enzyme system for serine synthesis, 5 μmoles of formaldehyde/ml. will combine with tetrahydroPGA/min. at 20°, or about 16 μmoles/ml./min. at 37°. As this is far in excess of any rates of enzymic serine synthesis attained *in vitro*, the data are consistent with the hypothesis that non-enzymic combination of tetrahydroPGA and formaldehyde precedes enzymic reaction of the product with glycine during the enzymic synthesis of serine.

When serine transhydroxymethylase was added to the reaction mixture the rate of combination of formaldehyde and tetrahydroPGA was diminished rather than increased (Table 3).

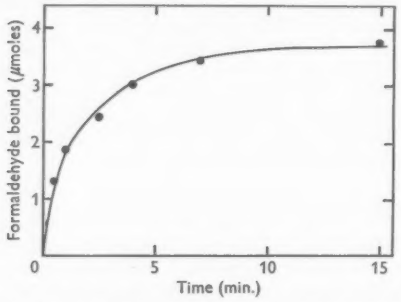


Fig. 1. Rate of combination of formaldehyde with tetrahydroPGA. At zero time tetrahydroPGA (final concentration 0·4 mM) was added to a solution of formaldehyde (0·4 mM) in potassium phosphate buffer, pH 7·2 (25 mM), containing ascorbic acid (2·5 mM). Total volume, 10 ml. The reaction was terminated by addition of charcoal and the mixture immediately treated as described in the Methods section.

Table 3. *Effect of serine transhydroxymethylase on binding of formaldehyde by tetrahydropteroylglutamic acid*

The reaction was performed in conical flasks; each contained formaldehyde (0·4 mM), ascorbic acid (5 mM) and potassium phosphate buffer (0·1 M, pH 7·2) in a final volume of 10 ml. Where enzyme was present 0·30 ml. (1·08 mg. of protein) of a preparation of specific activity 12 (μmoles of serine/mg. of protein/hr.) was employed. The reaction was started by addition of tetrahydroPGA (final concentration 0·4 mM) and terminated by shaking with 400 mg. of charcoal. Other conditions were as described in the Methods section.

Time (min.)	Formaldehyde disappearance (μmoles)	
	No enzyme	With enzyme
0·5	2·4	1·3
1·0	2·7	2·0

Rate of reaction of other hydropteridines with formaldehyde

Because of the smaller amounts of formaldehyde bound by other hydropteridines, measurement of their reaction rates was more difficult, but from Table 4 it may be seen that at 20° the reaction was substantially complete in 0·5 min. when the reactants were present at concentrations between 0·1 and 2·5 mM.

The rate of reaction of 4-hydroxy-6-methyltetrahydropteridine with formaldehyde is shown in more detail in Fig. 2. This figure shows that 4 mM-pteridine reached equilibrium with 0·4 mM-formaldehyde in 2–5 min. and that the reaction velocity is therefore of the same order as for the reaction between 0·4 mM-formaldehyde and 0·4 mM-tetrahydroPGA.

Table 4. *Rate of reaction of dihydropteroylglutamic acid and N^{10}-formyltetrahydropteroylglutamic acid with formaldehyde*

The pteridine was caused to react with 0·4 mM-HCHO in a total volume of 10 ml. under standard conditions as described in the Methods section.

Hydropteridine	Concn. (mM)	Formaldehyde disappearance (μmoles)	
		0·5 min.	15 min.
DihydroPGA	1·0	1·6	1·6
DihydroPGA	2·5	2·2	2·6
N^{10}-FormyltetrahydroPGA	1·0	1·4	1·7

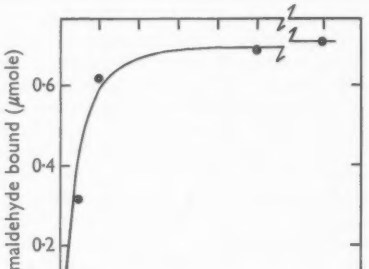


Fig. 2. Rate of combination of formaldehyde with 4-hydroxy-6-methyltetrahydropteridine. At zero time the pteridine (final concentration 4 mM) was added to a solution of formaldehyde (0·4 mM) in potassium phosphate buffer, pH 7·2 (0·1 M). Total volume, 10 ml. The reaction was terminated by addition of charcoal and the mixture immediately treated as described in the Methods section.

Enzyme activation by tetrahydro-, dihydro- and N^{10}-formyltetrahydro-pteroylglutamic acid

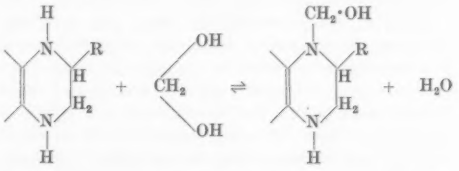
The very great difference between the dissociation constant of the compound formed by tetrahydroPGA and those of the compounds formed by dihydroPGA and N^{10}-formyltetrahydroPGA is surprising in view of previous results indicating comparable enzyme activation by tetrahydroPGA and dihydroPGA and appreciable activation even by N^{10}-formyltetrahydroPGA (Blakley, 1957*b*). It was therefore decided to reinvestigate the Michaelis constants for these compounds with N^{10}-formyltetrahydroPGA prepared from the highly purified N^{10}-formylpteroylglutamic acid and the more highly purified preparations of enzyme now available.

It was found that N^{10}-formyltetrahydroPGA prepared by hydrogenation of highly purified N^{10}-formylpteroylglutamic acid caused no measurable activation of the enzyme, so that activation previously obtained with crude N^{10}-formyltetrahydroPGA must be attributed to its contamination by tetrahydroPGA. DihydroPGA prepared by catalytic hydrogenation becomes contaminated with tetrahydroPGA if the reaction is allowed to proceed too long (Blakley, 1957*a*). When rigid precautions were taken to reduce such contamination of the dihydroPGA to a minimum, the activation of the enzymic synthesis of serine caused by the dihydroPGA was slight and variable, and frequently diminished as the enzymic reaction proceeded. Such results suggest that the activity of these dihydroPGA preparations was also due to traces of tetrahydroPGA which could easily become oxidized before or during the course of the enzymic reaction. As a check, dihydroPGA was synthesized by the reduction of pteroylglutamic acid with dithionite, as described by Futterman (1957). These preparations, which gave a spectrum identical with that of the material produced by catalytic hydrogenation, caused no detectable activation of the enzymic synthesis of serine.

It may be concluded therefore that tetrahydroPGA and its conjugates are the only compounds capable of activating the enzymic synthesis of serine. Re-determination of the Michaelis constant for tetrahydroPGA gave the value of $1{\cdot}1 \times 10^{-4}$ M, in fairly good agreement with previous results (Blakley, 1957*b*).

DISCUSSION

It has been established that formaldehyde in dilute solution reacts with 4-hydroxy-6-methyltetrahydropteridine, 2-amino-4-hydroxy-6-methyltetrahydropteridine, tetrahydroquinoxaline and N^{10}-formyltetrahydropteroylglutamic acid. It seems highly probable that the products of the combination of formaldehyde with these compounds are the corresponding N^5-hydroxymethyl derivatives. Each of these bases binds only a relatively small proportion of formaldehyde even when equimolecular proportions of formaldehyde and base are allowed to react, suggesting equilibria of the type:



The corresponding dissociation constants defined by

$$K = [\text{HCHO}]\,[\text{base}]/[\text{product}]$$

are of the order of 0·01–0·04. Attainment of equilibrium is relatively rapid, being complete in a few minutes when the reactants are present at concentrations of 0·1–1·0 mM. As expected on this formulation, N^5-formyl derivatives of hydropteridines bound little formaldehyde.

The reaction of 5 mM-formaldehyde with an equimolar concentration of tetrahydroPGA approaches much nearer to completion than does the reaction of formaldehyde with 6-methylhydropteridines or N-10-substituted tetrahydroPGA at similar concentrations. The reason for this cannot be ascertained with certainty from the data presented above, although they exclude the possibility that it is due to the binding of more than one molecule of formaldehyde by each molecule of tetrahydroPGA. Even when a fourfold excess of formaldehyde was added to solutions of tetrahydroPGA only approximately one molecular proportion of formaldehyde was removed from the solution. Furthermore, when N^5-formyltetrahydroPGA (leucovorin) was treated with formaldehyde, very little binding of formaldehyde occurred, indicating that no other grouping reacts as readily with formaldehyde as does the N-5-amine group.

The suggestion made some years ago (Blakley, 1954*b*) that tetrahydroPGA might react with formaldehyde to form N^5N^{10}-methylenetetrahydroPGA (II) has been currently revived by several authors to explain the unusual reactivity of tetrahydroPGA towards formaldehyde. The great difference between the binding of formaldehyde by tetrahydroPGA and the binding by 4-hydroxy-6-methyltetrahydropteridine and N^5- or N^{10}-formyltetrahydroPGA is emphasized by the results presented above. The dissociation constant for the product of the interaction of tetrahydroPGA and formaldehyde is approx. 4×10^{-4}, in contrast with

the constants for the compounds of the other hydropteridines mentioned, which have values of 0·03, 0·4 and 0·01 respectively. Since tetrahydroPGA binds one molecular proportion of formaldehyde under the conditions employed, and since formylation at N-5 or N-10 profoundly affects the reaction, the product must be N^5-hydroxymethylPGA (III), N^{10}-hydroxymethylPGA or N^5N^{10}-methylenetetrahydroPGA (II).

If N^5-hydroxymethylPGA were the product, however, the marked influence of formylating N-10 would be difficult to explain. It is unlikely that electromeric effects due to the N-10-formyl group could be transmitted past carbon atoms 6 and 9 to alter the reactivity of N-5 towards formaldehyde, and models indicate that the N-10-formyl group should not cause steric hindrance of the reaction of formaldehyde at N-5. Moreover, if the product were N^5-hydroxymethyltetrahydroPGA there is a large unexplained discrepancy between the dissociation constant (4×10^{-4}) for this compound and that for N^5-hydroxymethyl 4-hydroxy-6-methyltetrahydropteridine (0·03). Similar difficulties occur when N^{10}-hydroxymethylPGA is considered as the product. If the product is N^5N^{10}-methylenetetrahydroPGA, then formylation of either N-5 or N-10 might have the marked effect observed, since only hydroxymethyl-group formation is possible after formylation. It must be assumed that it is formation of the methylene bridge which produces a compound considerably more stable than any of the hydroxymethyl derivatives of tetrahydroPGA or other hydropteridines.

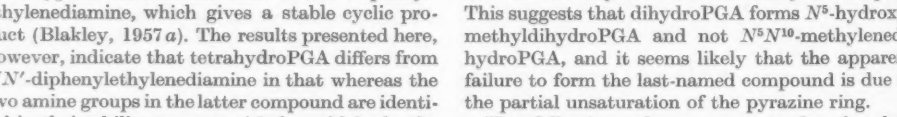
Yet the N^5N^{10}-methylene derivative cannot be completely stable as originally considered (Blakley, 1954*b*).

It was originally proposed that in its reaction with formaldehyde tetrahydroPGA might behave as a typical 1:2-diamine such as *NN'*-diphenylethylenediamine, which gives a stable cyclic product (Blakley, 1957*a*). The results presented here, however, indicate that tetrahydroPGA differs from *NN'*-diphenylethylenediamine in that whereas the two amine groups in the latter compound are identical in their ability to react with formaldehyde, the N-5-and N-10-amine groups of tetrahydroPGA show considerable difference. Thus when N-10 of tetrahydroPGA is formylated, the N-5-amine group combines with formaldehyde to yield a complex with a dissociation constant of 0·01: but when N-5 is formylated the derivative (leucovorin) reacts with formaldehyde to give a product with a very high dissociation constant (0·4). If N^5N^{10}-methylenetetrahydroPGA is formed by reaction of formaldehyde with tetrahydroPGA it might be expected therefore that the lability of the methylene–N-10 bond would render it less stable than the compound formed by *NN'*-diphenylethylenediamine.

Dissociation of the product of the reaction of tetrahydroPGA with formaldehyde is clearly implied by the earlier results on the instability of tetrahydroPGA after treatment with several molecular proportions of formaldehyde (Blakley, 1957*a*). Although the results reported above indicate that it is possible for only a relatively small amount of tetrahydroPGA to be present in the equilibrium mixture, this appears to be sufficient to allow an appreciable rate of oxidation of tetrahydroPGA, which in turn results in the dissociation of more of the formaldehyde complex. This provides an explanation for earlier results on the loss of coenzyme activity by tetrahydroPGA solutions during aeration. In the presence of formaldehyde the rate of coenzyme destruction became extremely slow when most of the coenzyme activity had been lost, whereas in the absence of formaldehyde destruction of coenzyme activity quickly proceeded to completion (Blakley, 1957*a*). This is interpreted as being due to the fact that in the presence of a constant amount of formaldehyde the tetrahydroPGA concentration was progressively diminished both by oxidation and by a relative increase in the amount combined with formaldehyde. Eventually the tetrahydroPGA concentration became so low that oxidation was at a negligible level.

The dissociation constant for the product of the reaction of dihydroPGA and formaldehyde is very similar to that for hydropteridines which form N^5-hydroxymethyl derivatives and much greater than for the tetrahydroPGA–formaldehyde complex. This suggests that dihydroPGA forms N^5-hydroxymethyldihydroPGA and not N^5N^{10}-methylenedihydroPGA, and it seems likely that the apparent failure to form the last-named compound is due to the partial unsaturation of the pyrazine ring.

The following scheme accounts for the data reported here and elsewhere:



It was found that serine transhydroxymethylase preparations did not catalyse the combination of formaldehyde and tetrahydroPGA, and that the latter non-enzymic reaction proceeded much faster than the enzymic synthesis of serine under the

same conditions of temperature, pH and concentration of reactants. Although it cannot be decided from the available data whether it is N^5-hydroxymethyltetrahydroPGA or N^5N^{10}-methylenetetrahydroPGA which participates in the enzymic synthesis of serine, a simple mechanism would be involved if N^5-hydroxymethyltetrahydroPGA were the active form.

SUMMARY

1. 4-Hydroxy-6-methyltetrahydropteridine has been synthesized and shown to bind formaldehyde in dilute solution. The evidence indicates a reversible reaction, the dissociation constant for the product being about 0·03.

2. 2-Amino-4-hydroxy-6-methyltetrahydropteridine and tetrahydroquinoxaline react similarly with formaldehyde, the corresponding dissociation constants being 0·04 and 0·01 respectively.

3. It is concluded that such hydropteridines undergo a reversible reaction with formaldehyde to form N^5-hydroxymethyl derivatives. This conclusion is strengthened by the fact that there is little binding of formaldehyde by N^5-formyl-4-hydroxy-6-methyltetrahydropteridine.

4. With 4-hydroxy-6-methyltetrahydropteridine, equilibrium is reached in a few minutes at room temperature when the initial concentration of pteridine is 4 mM and the initial concentration of formaldehyde is 0·5 mM.

5. Tetrahydropteroylglutamic acid binds 0·7–0·95 molecular proportion of formaldehyde, corresponding to the formation of a product with a dissociation constant of 4×10^{-4} or lower, the reaction being completed in 5–7 min. when the reactants have an initial concentration of 0·4 mM.

6. There is no detectable binding of formaldehyde by N^5-formyltetrahydropteroylglutamic acid, but the dissociation constants for the compounds formed by N^{10}-formyltetrahydropteroylglutamic acid and dihydropteroylglutamic acid were found to be 0·01.

7. A new procedure has been developed for preparation of serine transhydroxymethylase. The more highly purified enzyme obtained by this method has been used to re-determine the Michaelis constant for tetrahydropteroylglutamic acid. Dihydropteroylglutamic acid and N^{10}-formyltetrahydropteroylglutamic acid did not activate the enzyme.

8. The non-enzymic combination of formaldehyde and tetrahydropteroylglutamic acid proceeded so rapidly that its rate greatly exceeded the maximum rate of enzymic serine synthesis obtained under the same conditions *in vitro*. It may therefore be assumed that non-enzymic combination of formaldehyde and tetrahydropteroylglutamic acid is the first step in serine synthesis. Serine transhydroxymethylase preparations did not accelerate combination of formaldehyde and tetrahydropteroylglutamic acid.

9. It is concluded that tetrahydropteroylglutamic acid and formaldehyde react reversibly, possibly forming N^5N^{10}-methylenetetrahydropteroylglutamic acid. In view of the weakness with which N-10 of tetrahydropteroylglutamic acid binds formaldehyde, N^5-hydroxymethyltetrahydropteroylglutamic acid may be an intermediate in the formation and dissociation of N^5N^{10}-methylenetetrahydropteroylglutamic acid.

It is a pleasure to acknowledge my indebtedness to Dr H. P. Broquist and Dr D. J. Brown for the generous gifts of pteridines mentioned above and to Mr J. McKeough for skilled technical assistance.

REFERENCES

Albert, A., Brown, D. J. & Cheeseman, G. (1952). *J. chem. Soc.* p. 4219.
Blakley, R. L. (1954*a*). *Nature, Lond.*, **173**, 729.
Blakley, R. L. (1954*b*). *Biochem. J.* **58**, 448.
Blakley, R. L. (1955). *Biochem. J.* **61**, 315.
Blakley, R. L. (1957*a*). *Biochem. J.* **65**, 331.
Blakley, R. L. (1957*b*). *Biochem. J.* **65**, 342.
Blakley, R. L. (1957*c*). *Biochim. biophys. Acta*, **23**, 654.
Blakley, R. L. (1958). *Nature, Lond.*, **182**, 1719.
Brook, P. R. & Ramage, G. R. (1955). *J. chem. Soc.* p. 896.
Brown, D. J. (1952). *J. appl. Chem.* **2**, 239.
Brown, D. J. (1953). *J. chem. Soc.* p. 1644.
Futterman, S. (1957). *J. biol. Chem.* **228**, 1031.
Green, A. A. (1933). *J. Amer. chem. Soc.* **55**, 2331.
Kisliuk, R. L. (1957). *J. biol. Chem.* **227**, 805.
Kisliuk, R. L. & Sakami, W. (1954). *J. Amer. chem. Soc.* **76**, 1456.
Merz, V. & Ris, C. (1887). *Ber. dtsch. chem. Ges.* **20**, 1190.
Nash, T. (1953). *Biochem. J.* **55**, 416.
Osborne, M. G., Vercamer, E. N., Talbert, P. T. & Huennekens, F. M. (1957). *J. Amer. chem. Soc.* **79**, 6565.
Pohland, A., Flynn, E. H., Jones, R. G. & Shive, W. (1951). *J. Amer. chem. Soc.* **73**, 3247.

Nucleoside Diphosphokinase of Pea Seeds

By RUTH J. A. KIRKLAND
Department of Biochemistry, University of Sydney, Australia
AND J. F. TURNER
Plant Physiology Unit, Division of Food Preservation and Transport, C.S.I.R.O., Botany School, University of Sydney, Australia

(*Received* 22 *December* 1958)

The nucleoside diphosphokinase reaction may be formulated as involving a phosphate transfer from adenosine triphosphate to uridine diphosphate with the formation of uridine triphosphate and adenosine diphosphate:

Adenosine triphosphate + uridine diphosphate ⇌ adenosine diphosphate + uridine triphosphate (1)

Nucleoside diphosphokinase was demonstrated by Berg & Joklik (1953) in yeast extracts and these authors also observed that phosphate transfer occurred between adenosine triphosphate and inosine diphosphate. Krebs & Hems (1953) reported a reaction between adenosine triphosphate and inosine diphosphate giving adenosine diphosphate and inosine triphosphate in extracts from pigeon-breast muscle and rat intestinal mucosa. Berg & Joklik (1954) investigated in more detail the properties of the enzyme from yeast and rabbit muscle.

Indications of nucleoside diphosphokinase activity in higher plants were obtained by Burma & Mortimer (1956) in homogenates of sugar-beet leaves, and the presence of the enzyme in pea-seed extracts was suggested by Turner (1957) after investigations of sucrose synthesis. Nucleoside diphosphokinase activity has recently been observed in crude homogenates of leaves of *Impatiens holstii* (Ganguli, 1958) and in extracts of wheat-seedling scutella (Keys, 1958).

It is desirable that more detailed information should be available on the mechanisms of uridine triphosphate formation in higher plants. In the present investigation nucleoside diphosphokinase was found in pea-seed extracts and a preparation free from interfering enzyme reactions was obtained. Some properties of the enzyme and the equilibrium of the reaction were studied. Nucleoside diphosphokinase activity was also demonstrated in extracts from a number of other plant materials.

MATERIALS AND METHODS

Substrates. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine 5′-monophosphate (AMP), uridine triphosphate, uridine 5′-monophosphate, inosine triphosphate and inosine monophosphate were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A.

Uridine diphosphate, guanosine triphosphate and cytidine triphosphate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Preparation of nucleoside diphosphokinase from pea seeds. Crude extracts were prepared from dried pea seeds (*Pisum sativum* L.) as described previously (Turner, 1957) and centrifuged at 20 000 *g* for 20 min. at room temperature. The supernatant was treated with saturated $(NH_4)_2SO_4$, pH 7, at 4°, and the fraction precipitating between 45 and 55% saturation was dissolved in 7 ml. of water and dialysed with rocking at 4° for 2 hr. against 25 mM-phosphate (KH_2PO_4–Na_2HPO_4) buffer, pH 7.

The nucleoside diphosphokinase preparation was clear and colourless and could be stored at −15° for 6 months with only slight loss of activity. The enzyme extract possessed high activity and was normally diluted 1000–1400-fold with 0·5 mM-phosphate (KH_2PO_4–Na_2HPO_4) buffer, pH 7, before use. No interfering enzyme reactions involving any of the components of the nucleoside diphosphokinase reaction were observed. Adenosine triphosphatase activity was not detected in the extract. Heating at 60°, 70° and 80° for 10 min. resulted in 6, 65 and 95% loss respectively of nucleoside diphosphokinase activity.

Preparation of enzyme extracts from other plant tissues. Seeds of wheat, barley, broad bean and pea were germinated and grown for 8–10 days in damp sand in light. The roots or shoots were excised, ground in 50 mM-$NaHCO_3$, and the extract was centrifuged and the supernatant treated with saturated $(NH_4)_2SO_4$. For wheat and barley shoots, fractions were collected between 40 and 55% saturation, and for pea shoots and roots and broad-bean shoots and roots fractions were taken between 45 and 55% saturation. The precipitates were suspended in water and dialysed as described previously.

Sugar-cane-stem cuttings were grown in damp sand at 30° for 12 days. The shoots and roots were blended with 50 mM-$NaHCO_3$. The fractions obtained between 35 and 70% saturation with $(NH_4)_2SO_4$ were taken up in water and dialysed.

Soaked wheat seeds, laminae of leaves of silver beet (*Beta vulgaris* L. var. *cicla* Moq.) and potato tubers were blended with 50 mM-$NaHCO_3$. The centrifuged extracts from silver-beet leaves and potato tubers were treated with saturated $(NH_4)_2SO_4$ and the fraction precipitating between 45 and 55% saturation was suspended in water and dialysed. The fraction collected from wheat seeds was that precipitating between 45 and 60% saturation.

Reaction mixtures. The reaction mixtures were maintained at 30°. For the assay of nucleoside diphosphokinase

activity the following components were mixed: 0·3 (approx.) μmole of ATP, 0·3 (approx.) μmole of uridine diphosphate, 0·38 μmole of $MgCl_2$, 7·5 μmoles of 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl buffer, pH 8, 0·05 ml. of enzyme (containing approx. 0·3 μg. of protein); total volume 0·15 ml. The reaction mixtures were incubated for 15 min. The enzyme concentration was adjusted so that not more than 30% of ATP was converted into ADP in 15 min.: under these conditions the rate of reaction was approximately constant during the incubation period.

Reaction mixtures of similar composition were employed for the determination of the equilibrium except that a more concentrated enzyme (equivalent to 2·1 μg. of protein/reaction mixture) was used. Samples were taken after incubation for 120 and 150 min.

Digest samples were inactivated by the addition of 2 vol. of ethanol.

Detection and estimation of nucleotides. The nucleotides were separated on paper chromatograms, eluted and estimated spectrophotometrically, substantially as previously described (Turner & Turner, 1958). The papers were developed for 18 hr. with *iso*butyric acid–aq. NH_3 soln.–water (66:1:33, by vol.) as solvent (Pabst Laboratories, 1956). Because of a small amount of residual *iso*butyric acid remaining on the chromatograms, the spot areas were eluted with 3 ml. of 50 mM-phosphate (KH_2PO_4–Na_2HPO_4) buffer, pH 7, rather than with water. A molar extinction coefficient of $15{\cdot}4 \times 10^3$ at 260 mμ was used for the adenosine compounds (Bock, Ling, Morell & Lipton, 1956) and 10×10^3 at 262 mμ for the uridine compounds (Ploeser & Loring, 1949).

Determination of protein. The protein content of the enzyme extracts was estimated by the spectrophotometric method of Warburg & Christian (1941).

RESULTS

Nucleoside diphosphokinase activity

Preliminary experiments established that there was no change in ATP, ADP, uridine tri- or diphosphate when these were incubated individually with the enzyme preparation. The reaction could be followed by determining the change in any one of the reactants (equation 1). Nucleoside diphosphokinase activity of the pea-seed enzyme as estimated by formation of ADP and also disappearance of ATP is shown in Fig. 1. In assays of nucleoside diphosphokinase activity, changes in the adenosine nucleotides were followed because of their greater molar extinction coefficient.

The reverse reaction (i.e. the formation of ATP and uridine diphosphate from ADP and uridine triphosphate) was shown by using mixtures of the following composition: 1·21 μmoles of ADP, 1·20 μmoles of uridine triphosphate, 1·52 μmoles of $MgCl_2$, 30·0 μmoles of tris-HCl buffer, pH 8, 0·2 ml. of enzyme (containing 1·8 μg. of protein); total volume 0·6 ml. Results analogous to those illustrated in Fig. 1 were obtained.

Equilibrium of the reaction. All four reactants (ADP, ATP, uridine tri- and di-phosphate) were estimated in determining the apparent equilibrium constant (K^1). The value of K^1, which is defined as

$$K^1 = \frac{[\text{Uridine triphosphate}].[\text{ADP}]}{[\text{Uridine diphosphate}].[\text{ATP}]},$$

was found to be 0·91 (mean of eight determinations) at pH 8, and with 2·5 mM-$MgCl_2$.

Variation of pH had only slight effect on the value of K^1. In the presence of 2·5 mM-$MgCl_2$, K^1

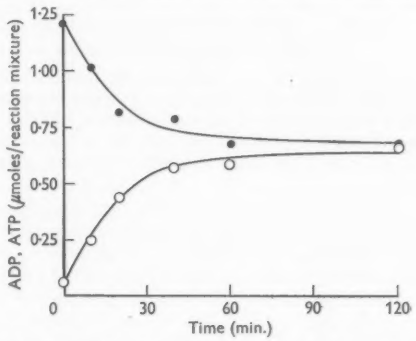


Fig. 1. Nucleoside diphosphokinase activity as measured by formation of ADP (○) and loss of ATP (●). The reaction mixture contained 1·27 μmoles of ATP, 1·24 μmoles of uridine diphosphate, 1·52 μmoles of $MgCl_2$, 30 μmoles of tris-HCl buffer, pH 8, 0·2 ml. of enzyme (containing 1·8 μg. of protein); total volume 0·6 ml. Temp. 30°.

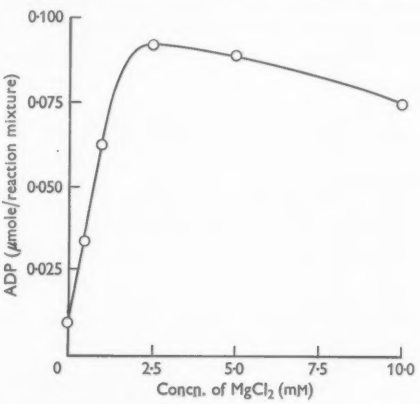


Fig. 2. Effect of $MgCl_2$ concentration on nucleoside diphosphokinase activity. The reaction mixtures contained 0·34 μmole of ATP, 0·36 μmole of uridine diphosphate, 7·5 μmoles of tris-HCl buffer, pH 8, 0·05 ml. of enzyme (containing 0·23 μg. of protein); total volume 0·15 ml. Concentration of $MgCl_2$ was varied as shown. Time of incubation, 15 min. Temp. 30°.

was 1·2, 0·91 and 0·81 at pH 7, 8 and 9 respectively. The value of K^1 was not significantly affected by variation of Mg^{2+} ion concentration. The values at pH 8 and with $MgCl_2$ concentrations 1·0, 2·5 and 5·0 mM were 0·96, 0·91 and 0·98 respectively.

Cofactor requirements. The enzyme was largely inactive in the absence of a suitable bivalent metal ion. Fig. 2 shows the effect of Mg^{2+} ion concentration on nucleoside diphosphokinase activity. The reaction rate reached a maximum at a concentration of 2·5 mM-$MgCl_2$; at concentrations greater than 5 mM, the rate decreased.

Mg^{2+} ions could be replaced by Mn^{2+}, Co^{2+} and Ca^{2+} ions and to a lesser extent by Zn^{2+} and Ni^{2+} ions (Table 1).

Effect of pH. The activity of the enzyme was studied in a series of buffers containing tris (final concentration 0·2M) and acetic acid over the range pH 6–9 (Fig. 3). The optimum pH was approximately 8. In separate experiments with glycine (final concn. 0·2M)–NaOH buffers the activity was observed to decrease by 10% when the pH was increased from 9 to 10.

Effect of inhibitors. Ethylenediaminetetra-acetate (EDTA) (10 mM) completely inhibited the reaction and mM-EDTA inhibited nucleoside diphosphokinase activity by 50%. Arsenate (10 mM) inhibited the reaction by 70%. Fluoride, iodoacetate or inorganic pyrophosphate at 10 mM-concentration, *p*-chloromercuribenzoate or molybdate at mM-concentration, or Hg^{2+} ions at 0·1 mM-concentration did not affect the activity.

Table 1. *Effect of bivalent metal ions on nucleoside diphosphokinase activity*

The reaction mixtures contained: 0·30 μmole of ATP, 0·31 μmole of uridine diphosphate, 7·5 μmoles of tris-HCl buffer, pH 8, 0·05 ml. of enzyme (containing 0·23 μg. of protein); total volume 0·15 ml. Salts of bivalent metals were added as shown. Time of incubation, 15 min. Temp. 30°.

Addition	Final concn. (mM)	ADP formed (μmole/reaction mixture)
None	—	0·003
$MgCl_2$	2·5	0·084
$MnCl_2$	0·5	0·043
	1·0	0·068
	2·5	0·064
$CoCl_2$	1·0	0·027
	2·5	0·061
$CaCl_2$	1·0	0·028
	2·5	0·053
$ZnSO_4$	1·0	0·023
	2·5	0·022
$NiCl_2$	2·5	0·022

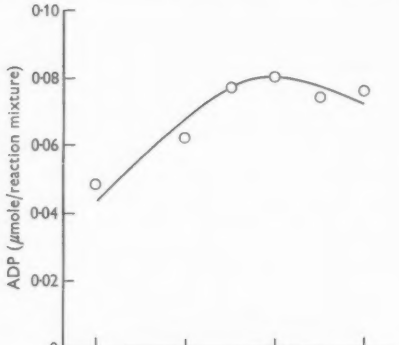


Fig. 3. Effect of pH on nucleoside diphosphokinase activity. The reaction mixtures contained 0·28 μmole of ATP, 0·26 μmole of uridine diphosphate, 0·38 μmole of $MgCl_2$, 7·5 μmoles of tris–acetate buffer of required pH, 0·05 ml. of enzyme (containing 0·35 μg. of protein); total volume 0·15 ml. Time of incubation, 15 min. Temp. 30°.

Specificity of the pea-seed nucleoside diphosphokinase preparation

When ADP was incubated with inosine triphosphate, guanosine triphosphate or cytidine triphosphate, ATP was formed together with the corresponding nucleoside diphosphate. The rates of reaction of these nucleoside triphosphates and that of uridine triphosphate are compared in Table 2. The reaction with inosine triphosphate was more rapid than with uridine triphosphate, whereas the rates with guanosine triphosphate and cytidine triphosphate were lower. In separate experiments it was found that there was no reaction when ATP was incubated with uridine 5′-monophosphate or inosine monophosphate.

Distribution of nucleoside diphosphokinase

All plant tissues examined yielded extracts containing nucleoside diphosphokinase activity. The tissues were wheat seeds and shoots, pea roots and

Table 2. *Specificity of the nucleoside diphosphokinase preparation*

The reaction mixtures contained 0·29 μmole of ADP, 0·38 μmole of $MgCl_2$, 7·5 μmoles of tris-HCl buffer, pH 8, 0·05 ml. of enzyme (containing 0·32 μg. of protein); total volume 0·15 ml. Nucleoside triphosphates were added as shown. Time of incubation, 20 min. Temp. 30°.

Nucleoside triphosphate	Amount added (μmole)	ATP formed (μmole/reaction mixture)
Uridine	0·30	0·052
Inosine	0·30	0·082
Guanosine	0·32	0·032
Cytidine	0·27	0·025

shoots, broad-bean roots and shoots, sugar-cane roots and shoots, barley shoots, silver-beet leaves and potato tubers. Extracts possessing very high nucleoside diphosphokinase activity were obtained from potato tubers.

DISCUSSION

The present study has shown that active nucleoside diphosphokinase preparations free from interfering enzymes may be readily obtained from pea seeds.

At the equilibrium of the reaction approximately equal amounts of ADP, ATP and uridine di- and tri-phosphate were present. The value of K^1 found was 0·91 at the optimum concentration (2·5 mM) of Mg^{2+} ions and at pH 8 and 30°. This confirms the conclusion of Berg & Joklik (1954) that the free energy of hydrolysis of the terminal pyrophosphate bond of uridine triphosphate is probably approximately equivalent to that of ATP. As pointed out by Noda, Kuby & Lardy (1954) and Turner & Turner (1958), values of K^1 in reactions of this type are not true thermodynamic-equilibrium constants but represent the total concentration of each substance (as ions and magnesium complexes) present at equilibrium. To calculate the true thermodynamic-equilibrium constant it would be necessary to determine the nature and amount of the components which actually participate in the reaction. The value of K^1 found in the present investigation for the nucleoside diphosphokinase reaction involving ADP, ATP and uridine di- and tri-phosphate is in agreement with the observation of Berg & Joklik (1954) that the equilibrium constant was approximately 1 with the enzyme from yeast.

The slight inhibitory effect of high Mg^{2+} ion concentration on the nucleoside diphosphokinase reaction may have been due to increased magnesium–nucleotide-complex formation. The optimum concentration of Mg^{2+} ions, 2·5 mM, is in reasonable agreement with that of 5 mM found by Berg & Joklik (1954) with the enzyme from yeast and rabbit-muscle extracts. These workers reported that Mn^{2+} and Ca^{2+} ions could replace Mg^{2+} ions and this observation is in accord with the present results. These results, together with the stimulation by Co^{2+}, Zn^{2+} and Ni^{2+} ions, suggest that the co-factor requirement of the enzyme from pea seeds is for a suitable bivalent cation.

The nucleoside diphosphokinase preparation from pea seeds catalysed a reaction between ADP and uridine triphosphate, inosine triphosphate, cytidine triphosphate or guanosine triphosphate. The reaction was most rapid with inosine triphosphate and uridine triphosphate; with cytidine triphosphate and guanosine triphosphate the rate of reaction was slower. There was no detectable reaction between ATP and uridine 5′-monophosphate or inosine monophosphate. These results support the suggestion of Berg & Joklik (1954) that the reaction is specific for a nucleoside triphosphate as phosphate donor and a nucleoside diphosphate as phosphate acceptor. As mentioned previously, Berg & Joklik reported a reaction between ADP and uridine triphosphate or inosine triphosphate with nucleoside diphosphokinase from yeast or rabbit muscle. Sanadi, Gibson, Ayengar & Jacob (1956) observed a reaction between ADP and guanosine triphosphate in extracts from pig-kidney cortex and ox-heart muscle.

The presence of nucleoside diphosphokinase in extracts of all plant tissues examined suggests a wide distribution for this enzyme. The enzymic formation of uridine triphosphate is of interest in relation to the carbohydrate metabolism of plants, as uridine diphosphate glucose may be formed from uridine triphosphate and glucose 1-phosphate by uridine diphosphate glucose pyrophosphorylase, which is also widely distributed in higher plants (Turner & Turner, 1958). It is known that uridine diphosphate glucose is the probable precursor of sucrose in plants (Cardini, Leloir & Chiriboga, 1955; Leloir & Cardini, 1953, 1955; Turner, 1954, 1957). The high nucleoside diphosphokinase activity of pea-seed extracts may be significant in relation to the rapid sucrose–starch conversions which occur in the developing pea seed (Turner & Turner, 1957; Turner, Turner & Lee, 1957). Similarly, the very active nucleoside diphosphokinase preparations obtained from potato tubers are consistent with the rapid sugar–starch transformations observed when potatoes are transferred from 0–1° to higher temperatures (Barker, 1936).

SUMMARY

1. Nucleoside diphosphokinase, which catalyses the reaction

$$\underset{\text{triphosphate}}{\text{adenosine}} + \underset{\text{diphosphate}}{\text{uridine}} \rightleftharpoons \underset{\text{diphosphate}}{\text{adenosine}} + \underset{\text{triphosphate}}{\text{uridine}}$$

was found in pea-seed extracts. Preparations free from interfering enzyme reactions were readily obtained.

2. The apparent equilibrium constant ([adenosine diphosphate] [uridine triphosphate]/[adenosine triphosphate] [uridine diphosphate]) was determined. Increase in Mg^{2+} ion concentration and variation in pH had little effect on the apparent equilibrium constant.

3. Mg^{2+} ions were necessary for the reaction. Mn^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+} and Ni^{2+} ions could partially replace Mg^{2+} ions as cofactor for the enzyme.

4. The effects of Mg^{2+} ion concentration, pH and inhibitors on nucleoside diphosphokinase activity were studied.

5. A rapid reaction occurred between adenosine diphosphate and inosine triphosphate or uridine triphosphate, whereas adenosine diphosphate and guanosine triphosphate or cytidine triphosphate reacted more slowly.

6. Extracts were prepared from a number of plant tissues and all yielded preparations containing nucleoside diphosphokinase activity.

7. The possible significance of the nucleoside diphosphokinase reaction in the carbohydrate metabolism of plants has been discussed.

This work was carried out during the tenure by one of us (R.J.A.K.) of a Studentship awarded by the Australian Commonwealth Scientific and Industrial Research Organization and of a University of Sydney Research Grant; support from these sources is gratefully acknowledged. The authors wish to express their indebtedness to Professor J. L. Still, Biochemistry Department, University of Sydney, Professor R. L. Crocker, Botany School, University of Sydney and Dr J. R. Vickery, Chief, Division of Food Preservation and Transport, C.S.I.R.O., in whose Laboratories the work was carried out.

REFERENCES

Barker, J. (1936). *Proc. Roy. Soc.* B, **119**, 453.
Berg, P. & Joklik, W. K. (1953). *Nature, Lond.*, **172**, 1008.
Berg, P. & Joklik, W. K. (1954). *J. biol. Chem.* **210**, 657.
Bock, R. M., Ling, N. S., Morell, S. A. & Lipton, S. H. (1956). *Arch. Biochem. Biophys.* **62**, 253.
Burma, D. P. & Mortimer, D. C. (1956). *Arch. Biochem. Biophys.* **62**, 16.
Cardini, C. E., Leloir, L. F. & Chiriboga, J. (1955). *J. biol. Chem.* **214**, 149.
Ganguli, N. C. (1958). *J. biol. Chem.* **232**, 337.
Keys, A. J. (1958). *Biochem. J.* **70**, 11P.
Krebs, H. A. & Hems, R. (1953). *Biochim. biophys. Acta*, **12**, 172.
Leloir, L. F. & Cardini, C. E. (1953). *J. Amer. chem. Soc.* **75**, 6084.
Leloir, L. F. & Cardini, C. E. (1955). *J. biol. Chem.* **214**, 157.
Noda, L., Kuby, S. A. & Lardy, H. A. (1954). *J. biol. Chem.* **210**, 83.
Pabst Laboratories (1956). Circular OR. 10. Milwaukee, Wis., U.S.A.
Ploeser, J. M. & Loring, H. S. (1949). *J. biol. Chem.* **178**, 431.
Sanadi, D. R., Gibson, D. M., Ayengar, P. & Jacob, M. (1956). *J. biol. Chem.* **218**, 505.
Turner, D. H. & Turner, J. F. (1957). *Aust. J. biol. Sci.* **10**, 302.
Turner, D. H. & Turner, J. F. (1958). *Biochem. J.* **69**, 448.
Turner, J. F. (1954). *Nature, Lond.*, **174**, 692.
Turner, J. F. (1957). *Biochem. J.* **67**, 450.
Turner, J. F., Turner, D. H. & Lee, J. B. (1957). *Aust. J. biol. Sci.* **10**, 408.
Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.

Index of Authors

Note. Entries marked with a section mark (§) refer to contributions to Symposia. Pages in *Proceedings of the Biochemical Society* are distinguished by P.

ABDEL-TAWAB, G. A., BRODA, E. & KELLNER, G. The production of pyruvic acid, oxaloacetic acid and α-oxoglutaric acid from glucose by tissue in culture 619

ABRAHAM, E. P. *see* SWALLOW, D. L. 326

ADINOLFI, A. *see* RUFFO, A. 613

AHRENS, E. H., Jun. *see* INSULL, W., Jun. 27

AMOORE, J. E., BARTLEY, W. & VAN HEYNINGEN, R. Distribution of sodium and potassium within cattle lens 126

ANDERSON, J. D., ANDREWS, P. & HOUGH, L. The incorporation of ^{14}C into the sorbitol of plum leaves 9P

ANDREWS, P. *see* ANDERSON, J. D. 9P

§ARIGONI, D. Steric aspects of the biosynthesis of terpenes and steroids 39P

ASTILL, B. D., FASSETT, D. W. & ROUDABUSH, R. L. The metabolism of 2:4:5-trihydroxybutyrophenone in the rat and dog 451

AUDLEY, B. G., GOODWIN, T. W. & MCEVOY, D. Flavinogenesis in *Candida flareri*; the role of urea and purines 8P

AVI-DOR, Y. & GONDA, O. Studies on the adenosine triphosphate–phosphate exchange and the hydrolysis of adenosine triphosphate catalysed by a particulate fraction from the mosquito 8

BACH, S. J. & HIBBITT, K. G. Biochemical aspects of bovine ketosis 87

BAILEY, R. W. Transglucosidase activity of rumen strains of *Streptococcus bovis*. 2. Isolation and properties of dextransucrase 42

BAILEY, R. W. & CLARKE, R. T. J. A bacterial dextranase 49

BAILIE, M. J. & CHRISTIE, G. S. The acute toxic action of dimethylnitrosamine on liver cells 473

BALÁZS, R. The point of the aerobic inhibition of glycolytic activity associated with brain mitochondria 561

BALDWIN, B. C., ROBINSON, D. & WILLIAMS, R. T. Ornithuric acid formation in hens 35P

BANGHAM, A. D. & DAWSON, R. M. C. The relation between the activity of a lecithinase and the electrophoretic charge of the substrate 486

BANGHAM, A. D. *see also* DAWSON, R. M. C. 493

§BARKER, G. R. Stereochemical specificities of some enzymes of nucleotide metabolism 42P

BARKER, G. R., LLOYD, J. B., MONTAGUE, M. D. & WOOD, N. F. The actions of some enzymes on purine and its derivatives 9P

§BARLOW, R. B. Steric aspects of drug action 40P

BARTLEY, W. *see* AMOORE, J. E. 126

BATCHELOR, A. *see* GOLBERG, L. 20P

BATES, C. M. *see* GOLBERG, L. 20P

BELL, D. *see* SMELLIE, R. M. S. 17P

BELL, D. J. & SILLER, W. G. Changes in erythrocyte chemistry accompanying temporary pectoral muscle lesions in young fowls 27P

BELL, D. J., SILLER, W. G. & CAMPBELL, J. G. Observations on 'cage layer fatigue' (CLF) in hens 32P

BELLAMY, D. The distribution of bound acetylcholine and choline acetylase in rat and pigeon brain 165

BILLIMORIA, J. D. & CURTIS, R. G. A study of butter phospholipid fractions 29P

BINNIE, B., HOLMS, W. H. & DAWES, E. A. Patterns of oxidative asimilation in *Sarcina lutea* 17P

BLAKLEY, R. L. The reaction of tetrahydropteroylglutamic acid and related hydropteridines with formaldehyde 707

BOND, G. *see* LEAF, G. 662

BOWEN, T. J., DAGLEY, S. & SYKES, J. A ribonucleoprotein component of *Escherichia coli* 419

BOYLAND, E., DAVIES, J. H. & WILLIAMS, K. Inhibition of β-glucuronidase by glucosaccharo-1→4-lactone 28P

BRANION, H. D. *see* MOTZOK, I. 177

BREUER, H. J. *see* WHITTAM, R. 638

BRODA, E. *see* ABDEL-TAWAB, G. A. 619

BUCHER, N. L. R. *see* POPJÁK, G. 34P

BU'LOCK, J. D. & GREGORY, H. The biosynthesis of polyacetylenes. 2. Origin of the carbon atoms 322

BURTON, P., KERR, L. M. H. & FRAZER, S. C. Demonstration of autoantibodies reacting with human-liver lactic dehydrogenase 12P

BUTTERY, S. H. *see* GALLAGHER, C. H. 575

BYRDE, R. J. W., DOWNING, D. F. & WOODCOCK, D. Fungal detoxication. 4. Metabolism of 2-methoxynaphthalene by *Aspergillus niger* 344

CALVIN, M. *see* MOSES, V. 19P

CAMPBELL, J. G. *see* BELL, D. J. 32P

CAMPBELL, P. N. & KERNOT, B. A. Incorporation of amino acids into the proteins of the isolated microsome fraction from duck livers 6P

CAMPBELL, P. N. *see also* GREENGARD, O. 305; RENDI, R. 34, 435

CHALLEN, S. B. *see* FAIRBAIRN, J. W. 556

CHEESMAN, D. F., KEELER, M. M. & STEN-KNUDSEN, O. The effects of adenosine triphosphate on the mechanical properties of surface films of L-myosin and actomyosin 357

CHRISTIE, G. S. *see* BAILIE, M. J. 473

CHU, E. *see* SCOTT, D. B. MCNAIR 426

CLARK, B. *see* HÜBSCHER, G. 7P

CLARKE, E. G. C. Micro-tests for sugars 13P

CLARKE, R. T. J. *see* BAILEY, R. W. 49

CLIFFE, E. E. & WALEY, S. G. Metabolism of γ-glutamyl peptides in subcellular fractions of rabbit liver 12P

COLLINS, F. D. Studies on phospholipids. 5. The separation of dinitrophenylated and methylated phospholipids by countercurrent distribution 281

COLLINS, F. D. Studies on phospholipids. 6. A complex phospholipid from sheep brain 532

COLPA-BOONSTRA, J. & HOLTON, F. A. The oxidation–reduction potential of cytochrome *b* in heart-muscle preparation 4P

CONNELL, G. E. & SMITHIES, O. Human haptoglobins: estimation and purification 115

COOTE, J. L. *see* REIS, P. J. 24P

CORNFORTH, R. H. *see* POPJÁK, G. 34P

CRAWFORD, M. A. *see* MILNE, M. D. 30P

CREASEY, W. A. & STOCKEN, L. A. The effect of ionizing radiation on nuclear phosphorylation in the radio-sensitive tissues of the rat 519

CROWDY, S. H., GREEN, A. P., GROVE, J. F., MCCLOSKEY, P. & MORRISON, A. The translocation of antibiotics in higher plants. 3. The estimation of griseofulvin relatives in plant tissue 230

CROWDY, S. H., GROVE, J. F. & MCCLOSKEY, P. The translocation of antibiotics in higher plants. 4. Systemic fungicidal activity and chemical structure in griseofulvin relatives 241

CRUMPTON, M. J. Identification of amino sugars 479
CUNNINGHAM, N. F. & MORTON, R. A. Unsaponifiable constituents of liver; ubiquinone and substance SC in various species 92
CURTIS, R. G. *see* BILLIMORIA, J. D. 29P

DAGLEY, S. *see* BOWEN, T. J. 419
DAVIDSON, J. N. *see* SMELLIE, R. M. S. 17P
DAVIES, J. H. *see* BOYLAND, E. 28P
DAVISON, A. N. *see* HOWELL, J. McC. 365
DAWES, E. A. *see* BINNIE, B. 17P
DAWKINS, M. J. R., JUDAH, J. D. & REES, K. R. The effect of chlorpromazine on the respiratory chain: cytochrome oxidase 204
DAWSON, R. M. C. & BANGHAM, A. D. The activation of surface films of lecithin by amphipathic molecules 493
DAWSON, R. M. C. *see also* BANGHAM, A. D. 486
DEWEY, D. L. & GREEN, F. O. Studies on the conversion of glucose into lactic acid in the Ehrlich ascites tumour 160
DICKENS, F. & WILLIAMSON, D. H. Studies on the metabolism of hydroxypyruvate in animal tissues 496
DIXON, G. H. & KORNBERG, H. L. Assay methods for key enzymes of the glyoxylate cycle 3P
DOWNING, D. F. *see* BYRDE, R. J. W. 344
DOY, C. H. & GIBSON, F. 1-(*o*-Carboxyphenylamino)-1-deoxyribulose: a compound formed by mutant strains of *Aerobacter aerogenes* and *Escherichia coli* blocked in the biosynthesis of tryptophan 586
DUNN, D. B. *see* SMITH, J. D. 294
DUNSTONE, J. R. Some cation-binding properties of cartilage 465

EDSON, N. L., HUNTER, G. J. E., KULKA, R. G. & WRIGHT, D. E. The metabolism of glycerol in *Mycobacterium butyricum* 249
EFRON, M. L. Two-way separation of amino acids and other ninhydrin-reacting substances by high-voltage electrophoresis followed by paper chromatography 691
ELLIOTT, T. H., PARKE, D. V. & WILLIAMS, R. T. Studies in detoxication. 79. The metabolism of *cyclo*[^{14}C]hexane and its derivatives 193

FAHMY, N. I. *see* MORTON, R. A. 99
FAIRBAIRN, J. W. & CHALLEN, S. B. The alkaloids of hemlock (*Conium maculatum* L.): distribution in relation to the development of the fruit 556
FASSETT, D. W. *see* ASTILL, B. D. 451
FESTENSTEIN, G. N. Substrate specificity of rumen cellulolytic enzymes 75
FRASER, M. J., SHIMIZU, H. & GUTFREUND, H. The reactions of amino acids with soluble ribonucleic acid from guinea-pig mammary cells 141
FRAZER, S. C. *see* BURTON, P. 12P
FUGMANN, U. *see* LUNDQUIST, F. 409

GALLAGHER, C. H. & BUTTERY, S. H. Biochemistry of sheep tissues: enzyme systems of liver, brain and kidney 575
GALTON, V. A. & PITT-RIVERS, R. A quantitative method for the separation of thyroid hormones and related compounds from serum and tissues with an anion-exchange resin 310
GALTON, V. A. & PITT-RIVERS, R. The identification of the acetic acid analogues of thyroxine and tri-iodothyronine in mammalian tissues 319
GALTON, V. A. & PITT-RIVERS, R. Thyroid-hormone metabolism in the kidney 314
GARDNER, I. C. *see* LEAF, G. 662
GIBBONS, R. A. Physical and chemical properties of mucoids from bovine cervical mucin 27P
GIBSON, F. *see* DOY, C. H. 586
GIRAO, C. B. *see* MILNE, M. D. 30P
GLASCOCK, R. F. & HOEKSTRA, W. G. Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep 673
GLOVER, J., GREEN, C. & STAINER, D. W. Sterol metabolism. 5. The uptake of sterols by organelles of intestinal mucosa and the site of their esterification during absorption 82
GLOVER, J. & STAINER, D. W. Sterol metabolism. 4. The absorption of 7-dehydrocholesterol in the rat 79
GOLBERG, L., MARTIN, L. E., BATES, C. M. & BATCHELOR, A. Stimulation of liver acid phosphatase activity following the parenteral injection of excessive doses of iron 20P
GONDA, O. *see* AVI-DOR, Y. 8
GOODWIN, T. W. *see* AUDLEY, B. G. 8P
GRAY, E. D. & SMELLIE, R. M. S. On the intramolecular location of deoxyribonucleotides incorporated into deoxyribonucleic acid by extracts of ascites tumour cells 16P
GRAY, E. D. *see also* SMELLIE, R. M. S. 17P
GRAYMORE, C. N. Hypersensitivity of the visual cell to iodoacetate 23P
GRAYMORE, C. N., TANSLEY, K. & KERLY, M. Metabolism of the developing retina. 2. The effect of an inherited retinal degeneration on the development of glycolysis in the rat retina 459
GREEN, A. L. & NICHOLLS, J. D. The reactivation of phosphorylated chymotrypsin 70
GREEN, A. P. *see* CROWDY, S. H. 230
GREEN, C. *see* GLOVER, J. 82
GREEN, F. O. *see* DEWEY, D. L. 160
GREENGARD, O. & CAMPBELL, P. N. Factors influencing the incorporation of amino acid into the protein of microsome and mitochondria preparations of rat liver and liver tumour 305
GREGORY, H. *see* BU'LOCK, J. D. 322
GREGORY, M. E. & HOLDSWORTH, E. S. A study of protein-binding in the metabolism of vitamin B_{12} 549
GROVE, J. F. *see* CROWDY, S. H. 230, 241
GUEST, J. R. The relationship between cobalamin and methionine synthesis in *Escherichia coli* PA 15 5P
GUTFREUND, H. The relation between amino acid activation and cell-sap RNA coupling 30P
GUTFREUND, H. *see also* FRASER, M. J. 141; HAMMOND, B. R. 349; JONES, E. A. 31P

HAMMOND, B. R. & GUTFREUND, H. The mechanism of ficin-catalysed reactions 349
HARRIS, H. The initiation of deoxyribonucleic acid synthesis in the connective-tissue cell, with some observations on the function of the nucleolus 54
HARRIS, H. *see also* WATTS, J. W. 147
HATCH, M. D. & TURNER, J. F. The aerobic inhibition of glycolysis in pea-seed extracts and its possible relationship to the Pasteur effect 524
HATHWAY, D. E. & SEAKINS, J. W. T. Hydroxystilbenes of *Eucalyptus wandoo* 369
HATHWAY, D. E. & SEAKINS, J. W. T. The occurrence of hydroxystilbenes in *Eucalyptus* 37P
HAUROWITZ, F. *see* HAWKINS, J. D. 5P
HAWKINS, J. D. & HAUROWITZ, F. Recovery of intravenously injected protein antigens from rat spleens 5P
HAWTHORNE, J. N. & HÜBSCHER, G. *myo*Inositol 2-phosphate, a constituent of liver monophosphoinositide 10P
HEATH, H. & HOARE, D. S. The biosynthesis of porphyrins from porphobilinogen by *Rhodopseudomonas spheroides* 14
HENDERSON, R. W., MORTON, R. K. & RAWLINSON, W. A. Oligosaccharide synthesis in the banana and its relationship to the transferase activity of invertase 340
HIBBITT, K. G. *see* BACH, S. J. 87
HICKS, R. M. The effect of isoniazid, propamidine, pentamidine and carbutamide on the growth and transaminases of *Escherichia coli* 22P
HILLER, O. *see* SMITHIES, O. 121

HOARE, D. S. *see* HEATH, H. 14
HOEKSTRA, W. G. *see* GLASCOCK, R. F. 673
HOLDSWORTH, E. S. *see* GREGORY, M. E. 549
HOLM-HANSEN, O. *see* MOSES, V. 19P
HOLMS, W. H. *see* BINNIE, B. 17P
HOLTON, F. A. *see* COLPA-BOONSTRA, J. 4P
HOPKINSON, L. & KERLY, M. The effect of monoiodoacetate on the aerobic metabolism of ox retina *in vitro* 22
HORNING, M. *see* POPJÁK, G. 34P
HOUGH, L. *see* ANDERSON, J. D. 9P
HOWELL, J. McC. & DAVISON, A. N. The copper content and cytochrome oxidase activity of tissues from normal and swayback lambs 365
HÜBSCHER, G. & CLARK, B. The isolation of phosphatidic acid from ox liver 7P
HÜBSCHER, G. *see also* HAWTHORNE, J. N. 10P
HUGGINS, A. K. *see* OTTAWAY, J. H. 25P
HUNT, A. L. Purification of the nicotinic acid hydroxylase system of *Pseudomonas fluorescens* KB1 1
HUNTER, G. J. E. *see* EDSON, N. L. 249

INSULL, W., Jun. & AHRENS, E. J., Jun. The fatty acids of human milk from mothers on diets taken *ad libitum* 27

JEFFREY, S. W. & SMITH, M. J. H. Some effects of salicylate on mitochondria from rat liver 462
JOCELYN, P. C. The effect of vitamin B_{12} on the reduction of gluthathione disulphide in human erythrocytes 11P
JOHNS, E. W. *see* PHILLIPS, D. M. P. 538
JONES, E. A. & GUTFREUND, H. Some properties of guinea-pig mammary gland mitochondria 31P
JONES, N. R. The estimation of anserinase activity by a low-temperature ninhydrin reaction 407
JUDAH, J. D. *see* DAWKINS, M. J. R. 204

KASBEKAR, D. K., LAVATE, W. V., REGE, D. V. & SREENIVASAN, A. A study of vitamin B_{12} protection in experimental liver injury to the rat by carbon tetrachloride 384
KASBEKAR, D. K., LAVATE, W. V., REGE, D. V. & SREENIVASAN, A. A study of vitamin B_{12} protection in experimental thyrotoxicosis in the rat 374
KASBEKAR, D. K. & SREENIVASAN, A. Biosynthesis of glutathione by rat erythrocytes 389
KEECH, D. B. *see* QUAYLE, J. R. 623, 631
KEELER, M. M. *see* CHEESMAN, D. F. 357
KEIR, H. M. Enzymic formation of deoxyribonucleoside triphosphates 17P
KEIR, H. M. *see also* SMELLIE, R. M. S. 17P
KELLNER, G. *see* ABDEL-TAWAB, G. A. 619
KERLY, M. *see* GRAYMORE, C. N. 459; HOPKINSON, L. 22
KERNOT, B. A. *see* CAMPBELL, P. N. 6P
KERR, L. M. H. *see* BURTON, P. 12P
KING, H. K. & LUCAS, N. Interaction between pyridoxal phosphate and amino acids 18P
KIRKLAND, R. J. A. & TURNER, J. F. Nucleoside diphosphokinase of pea seeds 716
KLÄNING, E. *see* LUNDQUIST, F. 409
§KLYNE, W. Stereochemical correlations 39P
KORNBERG, H. L. & LUND, P. Influence of growth substrates on levels of glyoxylate cycle enzymes in *Pseudomonas ovalis* Chester 33P
KORNBERG, H. L., PHIZACKERLEY, P. J. R. & SADLER, J. R. Synthesis of cell constituents from acetate by *Escherichia coli* 32P
KORNBERG, H. L. *see also* DIXON, G. H. 3P
KUHLMAN, R. E. & RESNIK, R. A. Enzymic studies of X-irradiated cornea and lens with special reference to glutathione reductase 261
KULKA, R. G. *see* EDSON, N. L. 249

LASCELLES, J. Adaptation to form bacteriochlorophyll in *Rhodopseudomonas spheroides*: changes in activity of enzymes concerned in pyrrole synthesis 508
LAVATE, W. V. *see* KASBEKAR, D. K. 374, 384
LEAF, G., GARDNER, I. C. & BOND, G. Observations on the composition and metabolism of the nitrogen-fixing root nodules of *Myrica* 662
LEVY, H. R. & POPJÁK, G. Liver mevalonic kinase 35P
LLOYD, A. G. Studies on sulphatases. 24. The use of barium chloranilate in the determination of enzymically liberated sulphate 133
LLOYD, J. B. *see* BARKER, G. R. 9P
LOUGHRIDGE, L. *see* MILNE, M. D. 30P
LOWE, C. U. *see* VENKATARAMAN, P. R. 430
LUCAS, N. *see* KING, H. K. 18P
LUND, P. *see* KORNBERG, H. L. 33P
LUNDIN, S. J. Acetylcholinesterase in goldfish muscles: studies on some substrates and inhibitors 210
LUNDQUIST, F., FUGMANN, U., KLÄNING, E. & RASMUSSEN, H. The metabolism of acetaldehyde in mammalian tissues: reactions in rat-liver suspensions under anaerobic conditions 409

MCCLOSKEY, P. *see* CROWDY, S. H. 230, 241
MCEVOY, D. *see* AUDLEY, B. G. 8P
MCINDOE, W. M. A lipophosphoprotein complex in hen plasma associated with yolk production 153
MCMULLEN, A. I. Nucleotides of *Hevea brasiliensis* latex: a ribonucleoprotein component 545
MANCHESTER, K. L. & YOUNG, F. G. Location of ^{14}C in protein from isolated rat diaphragm incubated *in vitro* with [^{14}C]amino acids and with $^{14}CO_2$ 136
MANDELSTAM, J. & ROGERS, H. J. The incorporation of amino acids into the cell-wall mucopeptide of staphylococci and the effect of antibiotics on the process 654
MARTIN, J. *see* OTTAWAY, J. H. 25P
MARTIN, L. E. *see* GOLBERG, L. 20P
MASSEY, V. On the physiological role of diaphorase 7P
MAW, G. A. Thetin–homocysteine transmethylase: the distribution of the enzyme, studied with the aid of trimethylsulphonium chloride as substrate 602
MEADOW, P. & WORK, E. Biosynthesis of diaminopimelic acid and lysine in *Escherichia coli*. 1. The incorporation of ^{14}C from various organic precursors into the diaminopimelic acid of a lysine-requiring mutant 396
MEADOW, P. & WORK, E. Biosynthesis of diaminopimelic acid and lysine in *Escherichia coli*. 2. Incorporation of [^{14}C]diaminopimelic acid, [^{14}C]lysine and [^{14}C]glucose 400
MERVYN, L. & MORTON, R. A. Unsaponifiable fraction of lipid from normal and diseased human kidney 106
MILNE, M. D., CRAWFORD, M. A., GIRÃO, C. B. & LOUGHRIDGE, L. The metabolic abnormality of Hartnup disease 30P
MITCHELL, P. & MOYLE, J. Succinyl-group translocation through the plasma membrane of *Micrococcus lysodeikticus* 21P
MONTAGUE, M. D. *see* BARKER, G. R. 9P
MORRISON, A. *see* CROWDY, S. H. 230
MORTON, R. A. & FAHMY, N. I. A study of 'unsaponifiable' extracts obtained from hydrolysates of pancreas and spleen 99
MORTON, R. A. *see also* CUNNINGHAM, N. F. 92; MERVYN, L. 106; PITT, G. A. J. 40P
MORTON, R. K. *see* HENDERSON, R. W. 340
MOSES, V., HOLM-HANSEN, O., PON, N. G., NISHIDA, K. & CALVIN, M. Photosynthetic uptake of labelled carbon dioxide by subcellular components of spinach leaves 19P
MOTZOK, I. Studies on alkaline phosphatases. 1. Kinetics of plasma phosphatase of normal and rachitic chicks 169

MOTZOK, I. & BRANION, H. D. Studies on alkaline phosphatases. 2. Factors influencing pH optima and Michaelis constant 177
MOYLE, J. *see* MITCHELL, P. 21P

NICHOLLS, J. D. *see* GREEN, A. L. 70
NISHIDA, K. *see* MOSES, V. 19P

OGSTON, A. G. & SHERMAN, T. F. Degradation of the hyaluronic acid complex of synovial fluid by proteolytic enzymes and by ethylenediaminetetra-acetic acid 301
OTTAWAY, J. H. Purification of growth hormone from pig pituitaries 22P
OTTAWAY, J. H., HUGGINS, A. K. & MARTIN, J. An apparatus for incubating tissue slices *in vitro* 25P

PALMER, D. E. & SOLOMON, J. B. Adenosine deaminase—a constitutive enzyme of the developing White Leghorn chick embryo 28P
PARKE, D. V. *see* ELLIOTT, T. H. 193
PAWAN, G. L. S. The possible conversion of fat into carbohydrate in the obese human 20P
PERKINS, H. R. & ROGERS, H. J. The products of the partial acid hydrolysis of the mucopeptide from cell walls of *Micrococcus lysodeikticus* 647
PERRY, S. V. & ZYDOWO, M. A ribonucleoprotein of skeletal muscle and its relation to the myofibril 682
PETERS, R. A. The inhibition of soluble aconitase by fluorocitric acid 11P
PHILLIPS, D. M. P. & JOHNS, E. W. A study of the proteinase content and the chromatography of thymus histones 538
PHIZACKERLEY, P. J. R. *see* KORNBERG, H. L. 32P
§PITT, G. A. J. & MORTON, R. A. *cis–trans* Isomers of retinene in visual processes 40P
PITT-RIVERS, R. *see* GALTON, V. A. 310, 314, 319
PON, N. G. *see* MOSES, V. 19P
POPJÁK, G., HORNING, M., BUCHER, N. L. R. & CORNFORTH, R. H. The formation of 'terpenoid' acids from mevalonic acid in liver enzyme preparations and their relation to sterol biosynthesis 34P
POPJÁK, G. *see also* LEVY, H. R. 35P
PUMPHREY, A. M. & REDFEARN, E. R. The rate of reduction of the endogenous ubiquinone in a heart-muscle preparation 2P

QUAYLE, J. R. & KEECH, D. B. Carbon assimilation by *Pseudomonas oxalaticus* (OX 1). 1. Formate and carbon dioxide utilization during growth on formate 623
QUAYLE, J. R. & KEECH, D. B. Carbon assimilation by *Pseudomonas oxalaticus* (OX 1). 2. Formate and carbon dioxide utilization by cell-free extracts of the organism grown on formate 631

RASMUSSEN, H. *see* LUNDQUIST, F. 409
RAWLINSON, W. A. *see* HENDERSON, R. W. 340
REDFEARN, E. R. *see* PUMPHREY, A. M. 2P
REES, K. R. *see* DAWKINS, M. J. R. 204
REGE, D. V. *see* KASBEKAR, D. K. 374, 384
REIS, P. J., COOTE, J. L. & WORK, T. S. Amino acid incorporation into proteins of isolated rat-liver mitochondria 24P
RENDI, R. & CAMPBELL, P. N. Amino acid incorporation into cell-free preparations of normal and neoplastic lymphatic tissues of the rat 34
RENDI, R. & CAMPBELL, P. N. The role of cytoplasmic ribonucleic acid in the incorporation of amino acids into microsomal proteins 435
RESNIK, R. A. *see* KUHLMAN, R. E. 261
RICHARDS, J. *see* SMELLIE, R. M. S. 17P
ROBERTS, J. J. & WARWICK, G. P. Studies on the metabolism of myleran (1:4-dimethanesulphonyloxybutane) 3P
ROBINSON, D. *see* BALDWIN, B. C. 35P
RODNIGHT, R. *see* SMITH, M. J. 1P
ROGERS, H. J. *see* MANDELSTAM, J. 654; PERKINS, H. R. 647
ROMANO, M. *see* RUFFO, A. 613
ROSEN, D. Intermolecular and intramolecular reactions of human-serum albumin after its X-irradiation in aqueous solution 597
ROSENBERG, H. The occurrence of guanidinoacetic acid and other substituted guanidines in mammalian liver 582
ROTSTEIN, J. & SCHUBERT, M. Some components of bovine nasal cartilage 29P
ROUDABUSH, R. L. *see* ASTILL, B. D. 451
ROY, A. B. Aryl sulphamate synthesis 19P
RUFFO, A., ROMANO, M. & ADINOLFI, A. Inhibition of aconitase by glyoxylate plus oxaloacetate 613

SADLER, J. R. *see* KORNBERG, H. L. 32P
SALTER, D. N. Purification and properties of a proteinase from *Bacillus cereus* NCTC 945 23P
SCHUBERT, M. *see* ROTSTEIN, J. 29P
SCOTT, D. B. MCNAIR & CHU, E. The oxidative pathway of carbohydrate metabolism in *Escherichia coli*. 6. Adaptation of glucose 6-phosphate dehydrogenase to growth in complex media 426
SEAKINS, J. W. T. *see* HATHWAY, D. E. 369, 37P
SETCHELL, B. P. Cerebral metabolism in the sheep. 1. Normal sheep 265
SETCHELL, B. P. Cerebral metabolism in the sheep. 2. Irreversible hypoglycaemic coma 275
SHELLABARGER, C. J. *see* TATA, J. R. 608
SHERMAN, T. F. *see* OGSTON, A. G. 301
SHIMI, I. R., SINGH, J. & WALKER, T. K. The component fatty acids of *Penicillium spinulosum* fat 184
SHIMIZU, H. *see* FRASER, M. J. 141
SILLER, W. G. *see* BELL, D. J. 27P, 32P
SINGH, J. *see* SHIMI, I. R. 184
§SLATER, E. C. Steric factors in enzyme action: oxidation–reduction enzymes 41P
SMELLIE, R. M. S., KEIR, H. M., GRAY, E. D., BELL, D., RICHARDS, J. & DAVIDSON, J. N. Net synthesis of deoxyribonucleic acid by extracts of ascites tumour cells 17P
SMELLIE, R. M. S. *see also* GRAY, E. D. 16P
SMITH, J. D. & DUNN, D. B. The occurrence of methylated guanines in ribonucleic acids from several sources 294
SMITH, M. J. & RODNIGHT, R. Chromatographic analysis of blood thiols 1P
SMITH, M. J. H. *see* JEFFREY, S. W. 462
SMITHIES, O. & HILLER, O. The genetic control of transferrins in humans 121
SMITHIES, O. *see also* CONNELL, G. E. 115
SOLOMON, J. B. *see* PALMER, D. E. 28P
SOUTHGATE, D. A. T. *see* WIDDOWSON, E. M. 200
SREENIVASAN, A. *see* KASBEKAR, D. K. 374, 384, 389
STAINER, D. W. *see* GLOVER, J. 79, 82
STEN-KNUDSEN, O. *see* CHEESMAN, D. F. 357
STEWART, H. B. & YOUNG, F. G. A substance from animal tissues which induces ketonuria 60
STICKINGS, C. E. Studies in the biochemistry of micro-organisms. 106. Metabolites of *Alternaria tenuis* auct.: the structure of tenuazoic acid 332
STOCKEN, L. A. *see* CREASEY, W. A. 519
SWALLOW, D. L. & ABRAHAM, E. P. The amide and carboxyl groups of bacitracin A 326
SYKES, J. *see* BOWEN, T. J. 419

TANSLEY, K. *see* GRAYMORE, C. N. 459
TATA, J. R. A new aspect of the interaction between thyroxine and proteins 222
TATA, J. R. An unusual property of thyroxine and other iodophenols 214
TATA, J. R. & SHELLABARGER, C. J. An explanation for the difference between the responses of mammals and birds to thyroxine and tri-iodothyronine 608

TAYLOR, W. Investigation of the products of metabolism after incubation of deoxycorticosterone with rabbit-liver homogenate 442
TAYLOR, W. H. Gastric proteolysis in diseases affecting the stomach 1P
TEALE, F. W. J. & WEBER, G. Ultraviolet fluorescence of proteins 15P
TEALE, F. W. J. *see also* WEBER, G. 15P
TURNER, J. F. *see* HATCH, M. D. 524; KIRKLAND, R. J. A. 716

VAN HEYNINGEN, R. *see* AMOORE, J. E. 126
VENKATARAMAN, P. R. & LOWE, C. U. Effect of ethanol on rat-liver ribonucleoprotein previously exposed to cold trichloroacetic acid 430
VILLANUEVA, J. R. Purification of a nitro-reductase enzyme of a *Nocardia* sp. 36P

WALEY, S. G. *see* CLIFFE, E. E. 12P
WALKER, J. R. L. Pyruvate metabolism in *Lactobacillus brevis* 188
WALKER, T. K. *see* SHIMI, I. R. 184
WARREN, F. L. & WHITTAKER, M. Molecular structure of glycogens isolated from the livers of foetal animals 288
WARWICK, G. P. *see* ROBERTS, J. J. 3P
WATTS, J. W. & HARRIS, H. Turnover of nucleic acids in a non-multiplying animal cell 147
§WEBB, E. C. Steric factors in enzyme action: hydrolytic enzymes 41P
WEBER, G. & TEALE, F. W. J. Polarization of the ultraviolet fluorescence and electronic energy transfer in proteins 15P
WEBER, G. *see also* TEALE, F. W. J. 15P
WEIDMANN, S. M. *see* WHITEHEAD, R. G. 667
WHITEHEAD, R. G. & WEIDMANN, S. M. Oxidative enzyme systems in ossifying cartilage 667
WHITTAKER, M. *see* WARREN, F. L. 288
WHITTAKER, V. P. The isolation and characterization of acetylcholine-containing particles from brain 694
WHITTAM, R. & BREUER, H. J. Ion transport and metabolism in slices of guinea-pig seminal-vesicle mucosa 638
WIDDOWSON, E. M. & SOUTHGATE, D. A. T. Haemorrhage and tissue electrolytes 200
WILLIAMS, K. *see* BOYLAND, E. 28P
WILLIAMS, R. T. *see* BALDWIN, B. C. 35P; ELLIOTT, T. H. 193
WILLIAMSON, D. H. *see* DICKENS, F. 496
WOOD, N. F. *see* BARKER, G. R. 9P
WOODCOCK, D. *see* BYRDE, R. J. W. 344
WORK, E. *see* MEADOW, P. 396, 400
WORK, T. S. *see* REIS, P. J. 24P
WRIGHT, D. E. *see* EDSON, N. L. 249

YOUNG, F. G. *see* MANCHESTER, K. L. 136; STEWART, H. B. 60

ZYDOWO, M. *see* PERRY, S. V. 682

Index of Subjects

Note. Entries marked with a section mark (§) refer to contributions to Symposia.
Pages in *Proceedings of the Biochemical Society* are distinguished by P.

Acetaldehyde, metabolism of, in rat-liver suspensions under anaerobic conditions (Lundquist, F., Fugmann, U., Kläning, E. & Rasmussen, H.) 409

Acetate, formation of, from pyruvate by *Lactobacillus brevis* (Walker, J. R. L.) 188

[1-^{14}C]Acetate, incorporation of, into polyacetylenes by a basidiomycete (Bu'Lock, J. D. & Gregory, H.) 322

Acetate, synthesis of cell constituents from, by *Escherichia coli* (Kornberg, H. L., Phizackerley, P. J. R. & Sadler, J. R.) 32P

α-Acetyl-γ-*sec.*-butyltetramic acid, identity of, with tenuazonic acid (Stickings, C. E.) 332

Acetylcholine, bound, distribution of, in rat and pigeon brain (Bellamy, D.) 165

Acetylcholine-containing particles from brain, isolation and characterization of (Whittaker, V. P.) 694

Acetylcholinesterase in goldfish muscles (Lundin, S. J.) 210

Aconitase, inhibition of, by glyoxylate plus oxaloacetate (Ruffo, A., Romano, M. & Adinolfi, A.) 613

Aconitase, soluble, inhibition of, by fluorocitric acid (Peters, R. A.) 11P

Actomyosin, effects of adenosine triphosphate on mechanical properties of surface films of (Cheesman, D. F., Keeler, M. M. & Sten-Knudsen, O.) 357

Adenine, exchange of, in ribonucleic acid of macrophages (Watts, J. W. & Harris, H.) 147

Adenosine deaminase—a constitutive enzyme of developing White Leghorn chick embryo (Palmer, D. E. & Solomon, J. B.) 28P

Adenosine triphosphate, effects of, on mechanical properties of surface films of L-myosin and actomysin (Cheesman, D. F., Keeler, M. M. & Sten-Knudsen, O.) 357

Adenosine triphosphate, studies on hydrolysis of, catalysed by respiratory particles from the mosquito (Avi-Dor, Y. & Gondi, O.) 8

Aedes aegypti see Mosquito

Aerobacter aerogenes, 1-(*o*-carboxyphenylamino)-1-deoxyribulose formed by mutant tryptophan-requiring strains of (Doy, C. H. & Gibson, F.) 586

Albumin, serum, human, intermolecular and intramolecular reactions of, after X-irradiation in aqueous solution (Rosen, D.) 597

Alkaloids of hemlock (Fairbairn, J. W. & Challen, S. B.) 556

Alternaria tenuis, metabolites of (Stickings, C. E.) 332

Amino acid activation, relation between cell-sap ribonucleic acid coupling and (Gutfreund, H.) 30P

Amino acid, incorporation of, into cell-free preparations of normal and neoplastic lymphatic tissues of the rat (Rendi, R. & Campbell, P. N.) 34

Amino acid, incorporation of, into protein of microsome and mitochondria preparations of rat liver and liver tumour (Greengard, O. & Campbell, P. N.) 305

Amino acid, incorporation of, into proteins of isolated rat-liver mitochondria (Reis, P. J., Coote, J. L. & Work, T. S.) 24P

Amino acids and other ninhydrin-reacting substances, two-way separation of, by high-voltage electrophoresis followed by paper chromatography (Efron, M. L.) 691

Amino acids, incorporation of, into cell-wall mucopeptide of staphylococci and effect of antibiotics on the process (Mandelstam, J. & Rogers, H. J.) 654

Amino acids, incorporation of, into proteins of isolated microsome fraction from duck liver (Campbell, P. N. & Kernot, B. A.) 6P

Amino acids, interaction between pyridoxal phosphate and (King, H. K. & Lucas, N.) 18P

[^{14}C]Amino acids, location of ^{14}C in protein from isolated rat diaphragm incubated with, and with $^{14}CO_2$ (Manchester, K. L. & Young, F. G.) 136

Amino acids, reactions of, with soluble ribonucleic acid from guinea-pig mammary cells (Fraser, M. J., Shimizu, H. & Gutfreund, H.) 141

Amino acids, role of cytoplasmic ribonucleic acid in incorporation of, into microsomal proteins (Rendi, R. & Campbell, P. N.) 435

Amino sugars, separation of, by paper chromatography, electrophoresis and ion-exchange (Crumpton, M. J.) 479

δ-Aminolaevulic acid, synthesis of, by *Rhodopseudomonas spheroides* (Lascelles, J.) 508

ε-(Aminosuccinyl)lysine, preparation of, from bacitracin A (Swallow, D. L. & Abraham, E. P.) 326

Amphipathic molecules, activation of surface films of lecithin by (Dawson, R. M. C. & Bangham, A. D.) 493

Anserinase activity, estimation of, by low-temperature ninhydrin reaction (Jones, N. R.) 407

Arginine, detection of, in rat liver (Rosenberg, H.) 582

Aryl sulphamate synthesis (Roy, A. B.) 19P

Ascites tumour cells, intramolecular location of deoxyribonucleotides incorporated into deoxyribonucleic acid by extracts of (Gray, E. D. & Smellie, R. M. S.) 16P

Ascites tumour cells, net synthesis of deoxyribonucleic acid by extracts of (Smellie, R. M. S., Keir, H. M., Gray, E. D., Bell, D., Richards, J. & Davidson, J. N.) 17P

Ascites tumour, Ehrlich, studies on the conversion of glucose into lactic acid in (Dewey, D. L. & Green, F. O.) 160

Aspergillus niger, metabolism of 2-methoxynaphthalene by (Byrde, R. J. W., Downing, D. F. & Woodcock, D.) 344

Autoantibodies, demonstration of, reacting with human-liver lactic dehydrogenase (Burton, P., Kerr, L. M. H. & Frazer, S. C.) 12P

Bacillus cereus, purification and properties of a proteinase from (Salter, D. N.) 23P

Bacitracin A, amide and carboxyl groups of (Swallow, D. L. & Abraham, E. P.) 326

Bacteriochlorophyll, adaptation to form, in *Rhodopseudomonas spheroides* (Lascelles, J.) 508

Banana, oligosaccharide synthesis in, and its relationship to the transferase activity of invertase (Henderson, R. W., Morton, R. K. & Rawlinson, W. A.) 340

Barium chloranilate, use of, in the determination of enzymically liberated sulphate (Lloyd, A. G.) 133

Basidiomycete, biosynthesis of polyacetylenes by a (Bu'Lock, J. D. & Gregory, H.) 322

Bean, estimation of griseofulvin relatives in tissues of (Crowdy, S. H., Green, A. P., Grove, J. F., McCloskey, P. & Morrison, A.) 230

Blood thiols, chromatographic analysis of (Smith, M. J. & Rodnight, R.) 1P

Brain, isolation and characterization of acetylcholine-containing particles from (Whittaker, V. P.) 694

Brain mitochondria, point of aerobic inhibition of glycolysis associated with (Balázs, R.) 561

Brain, rat and pigeon, distribution of bound acetylcholine and choline acetylase in (Bellamy, D.) 165
Brain, separation by countercurrent distribution of dinitrophenylated and methylated phospholipids from (Collins, F. D.) 281
Brain, sheep, complex phospholipid from (Collins, F. D.) 532
Brain, sheep, enzyme systems of (Gallagher, C. H. & Buttery, S. H.) 575
Butter, phospholipid fractions from (Billimoria, J. D. & Curtis, R. G.) 29P

'Cage layer fatigue' in hens, observations on (Bell, D. J., Siller, W. G. & Campbell, J. G.) 32P
Candida flareri, flavinogenesis in, role of urea and purines in (Audley, B. G., Goodwin, T. W. & McEvoy, D.) 8P
Carbohydrate, conversion of fat into, in the obese human (Pawan, G. L. S.) 20P
Carbon dioxide, radioactive ($^{14}CO_2$), location of ^{14}C in protein from isolated rat diaphragm incubated with [^{14}C]amino acids and (Manchester, K. L. & Young, F. G.) 136
Carbon dioxide, radioactive ($^{14}CO_2$), photosynthetic uptake of, by subcellular components of spinach leaves (Moses, V., Holm-Hansen, O., Pon, N. G., Nishida, K. & Calvin, M.) 19P
Carbon dioxide, utilization of, by cell-free extracts of *Pseudomonas oxalaticus* (OX 1) (Quayle, J. R. & Keech, D. B.) 631
Carbon dioxide, utilization of, by *Pseudomonas oxalaticus* (OX 1) (Quayle, J. R. & Keech, D. B.) 623
Carbon isotope (^{14}C), incorporation of, into diaminopimelic acid by *Escherichia coli* (Meadow, P. & Work, E.) 396
Carbon isotope (^{14}C), incorporation of, into sorbitol of plum leaves (Anderson, J. D., Andrews, P. & Hough, L.) 9P
Carbon isotope (^{14}C), location of, in protein from isolated rat diaphragm incubated with [^{14}C]amino acids and with $^{14}CO_2$ (Manchester, K. L. & Young, F. G.) 136
Carbon tetrachloride, vitamin B_{12} protection in experimental liver injury to the rat by (Kasbekar, D. K., Lavate, W. V., Rege, D. V. & Sreenivasan, A.) 384
Carboxydismutase, presence of, in *Pseudomonas oxalaticus* (OX 1) (Quayle, J. R. & Keech, D. B.) 631
1-(*o*-Carboxyphenylamino)-1-deoxyribulose formed by mutant strains of *Aerobacter aerogenes* and *Escherichia coli* blocked in the biosynthesis of tryptophan (Doy, C. H. & Gibson, F.) 586
Carbutamide, effect of, on growth and transaminases of *Escherichia coli* (Hicks, R. M.) 22P
Cartilage, cation-binding properties of (Dunstone, J. R.) 465
Cartilage, nasal, bovine, components of (Rotstein, J. & Schubert, M.) 29P
Cartilage, ossifying, oxidative enzyme systems in (Whitehead, R. G. & Weidmann, S. M.) 667
Cation-binding properties of cartilage (Dunstone, J. R.) 465
Cell wall, bacterial, incorporation of [^{14}C]diaminopimelic acid and [^{14}C]lysine into (Meadow, P. & Work, E.) 400
Cell wall, incorporation of amino acids into mucopeptide of (Mandelstam, J. & Rogers, H. J.) 654
Cell wall, products of partial acid hydrolysis of mucopeptide from (Perkins, H. R. & Rogers, H. J.) 647
Cellulolytic enzymes, rumen, substrate specificity of (Festenstein, G. N.) 75
Cerebrum, metabolism of, in normal sheep (Setchell, B. P.) 265
Chicks, normal and rachitic, kinetics of plasma phosphatase of (Motzok, I.) 169
Chlorpromazine, effect of, on the respiratory chain (Dawkins, M. J. R., Judah, J. D. & Rees, K. R.) 204
Cholesterol, intestinal absorption of, in the guinea pig (Glover, J., Green, C. & Stainer, D. W.) 82
Choline acetylase, distribution of, in rat and pigeon brain (Bellamy, D.) 165
Chymotrypsin, phosphorylated, reactivation of (Green, A. L. & Nicholls, J. D.) 70
Citric acid cycle in *Mycobacterium butyricum* (Edson, N. L., Hunter, G. J. E., Kulka, R. G. & Wright, D. E.) 249
Cobalamin and methionine synthesis in *Escherichia coli* (Guest, J. R.) 5P
Conium maculatum L. *see* Hemlock
Connective-tissue cell, initiation of deoxyribonucleic acid synthesis in (Harris, H.) 54
Copper, content of, in tissues from normal and swayback lambs (Howell, J. McC. & Davison, A. N.) 365
Cornea, X-irradiated, enzymic studies of, with special reference to glutathione reductase (Kuhlman, R. E. & Resnik, R. A.) 261
Cyanocobalamin, protein-bound, activity of, for microorganisms (Gregory, M. E. & Holdsworth, E. S.) 549
Cytochrome *b*, oxidation–reduction potential of, in heart-muscle preparation (Colpa-Boonstra, J. & Holton, F. A.) 4P
Cytochrome oxidase, activity of, in tissues from normal and swayback lambs (Howell, J. McC. & Davison, A. N.) 365
Cytochrome oxidase, effect of chlorpromazine on (Dawkins, M. J. R., Judah, J. D. & Rees, K. R.) 204

DDT *see* 1:1:1-Trichloro-2:2-di-(*p*-chlorophenyl)ethane
7-Dehydrocholesterol, absorption of, in rat (Glover, J. & Stainer, D. W.) 79
7-Dehydrocholesterol, intestinal absorption of, in the rat (Glover, J., Green, C. & Stainer, D. W.) 82
Deoxycorticosterone, investigation of products of metabolism after incubation of, with liver homogenate (Taylor, W.) 442
Deoxyribonucleic acid, initiation of, synthesis in the connective-tissue cell, with some observations on the function of the nucleolus (Harris, H.) 54
Deoxyribonucleic acid, intramolecular location of deoxyribonucleotides incorporated into, by extracts of ascites tumour cells (Gray, E. D. & Smellie, R. M. S.) 16P
Deoxyribonucleic acid, net synthesis of, by extracts of ascites tumour cells (Smellie, R. M. S., Keir, H. M., Gray, E. D., Bell, D., Richards, J. & Davidson, J. N.) 17P
Deoxyribonucleoside triphosphates, enzymic formation of (Keir, H. M.) 17P
Dextranase, activity of, in strains of *Lactobacillus bifidus* (Bailey, R. W. & Clarke, R. T. J.) 49
Dextransucrase, isolation and properties of (Bailey, R. W.) 42
[^{14}C]Diaminopimelic acid, incorporation of, by *Escherichia coli* (Meadow, P. & Work, E.) 400
Diaminopimelic acid, incorporation of ^{14}C from organic precursors into, of a lysine-requiring mutant (Meadow, P. & Work, E.) 396
Diaphorase, physiological role of (Massey, V.) 7P
Diaphragm, rat, isolated, incubated with [^{14}C]amino acids and with $^{14}CO_2$, location of ^{14}C in protein from (Manchester, K. L. & Young, F. G.) 136
20α:21-Dihydroxypregn-4-en-3-one and its 20β-epimer as metabolites of deoxycorticosterone (Taylor, W.) 442
6β:21-Dihydroxypregn-4-ene-3:20-dione as a metabolite of deoxycorticosterone (Taylor, W.) 442
3α:21-Dihydroxy-5α-pregnan-20-one and its 3β-epimer as metabolites of deoxycorticosterone (Taylor, W.) 442
3α:21-Dihydroxy-5β-pregnan-20-one as a metabolite of deoxycorticosterone (Taylor, W.) 442
1:4-Dimethanesulphonyloxybutane (myleran), studies on the metabolism of (Roberts, J. J. & Warwick, G. P.) 3P
2-Dimethyl-6-hydroxypurine, presence of, in ribonucleic acids (Smith, J. D. & Dunn, D. B.) 294

Dimethylnitrosamine, acute toxic action of, on liver cells (Bailie, M. J. & Christie, G. S.) 473
§Drug action, steric aspects of (Barlow, R. B.) 40P

Ehrlich ascites tumour, studies on the conversion of glucose into lactic acid in (Dewey, D. L. & Green, F. O.) 160
Electrolytes, tissue, and haemorrhage (Widdowson, E. M. & Southgate, D. A. T.) 200
§Enzymes, hydrolytic, steric factors in action of (Webb, E. C.) 41P
§Enzymes, oxidation–reduction, steric factors in action of (Slater, E. C.) 41P
Enzymes, proteolytic, degradation of the hyaluronic acid complex of synovial fluid by (Ogston, A. G. & Sherman, T. F.) 301
Erythrocyte chemistry accompanying temporary pectoral muscle lesions in young fowls, changes in (Bell, D. J. & Siller, W. G.) 27P
Erythrocytes, human, effect of vitamin B_{12} on reduction of glutathione disulphide in (Jocelyn, P. C.) 11P
Erythrocytes, rat, biosynthesis of glutathione by (Kasbekar, D. K. & Sreenivasan, A.) 389
Escherichia coli, adaptation of glucose 6-phosphate dehydrogenase to growth of, in complex media (Scott, D. B. McNair & Chu, E.) 426
Escherichia coli, 1-(*o*-carboxyphenylamino)-1-deoxyribulose formed by mutant tryptophan-requiring strains of (Doy, C. H. & Gibson, F.) 586
Escherichia coli, effect of isoniazid, propamidine, pentamidine and carbutamide on growth and transaminases of (Hicks, R. M.) 22P
Escherichia coli, incorporation of ^{14}C from organic precursors into diaminopimelic acid by lysine-requiring mutant of (Meadow, P. & Work, E.) 396
Escherichia coli, relationship between cobalamin and methionine synthesis in (Guest, J. R.) 5P
Escherichia coli, ribonucleoprotein component of (Bowen, T. J., Dagley, S. & Sykes, J.) 419
Escherichia coli, synthesis of cell constituents from acetate by (Kornberg, H. L., Phizackerley, P. J. R. & Sadler, J. R.) 32P
Ethanol, effect of, on rat-liver ribonucleoprotein exposed to cold trichloroacetic acid (Venkataraman, P. R. & Lowe, C. U.) 430
Ethylenediaminetetra-acetic acid, degradation of the hyaluronic acid complex of synovial fluid by (Ogston, A. G. & Sherman, T. F.) 301
Eucalyptus, occurrence of hydroxystilbenes in (Hathway, D. E. & Seakins, J. W. T.) 37P
Eucalyptus wandoo, hydroxystilbenes of (Hathway, D. E. & Seakins, J. W. T.) 369

Fat, component fatty acids of, from *Penicillium spinulosum* (Shimi, I. R., Singh, J. & Walker, T. K.) 184
Fat, conversion of, into carbohydrate in the obese human (Pawan, G. L. S.) 20P
Fatty acids, component, of *Penicillium spinulosum* fat (Shimi, I. R., Singh, J. & Walker, T. K.) 184
Fatty acids of human milk from mothers on diets taken *ad libitum* (Insull, W., Jun. & Ahrens, E. H., Jun.) 27
Fatty alcohol, isolation of, from the phospholipids of sheep brain (Collins, F. D.) 532
Ficin, mechanism of reactions catalysed by (Hammond, B. R. & Gutfreund, H.) 349
Fluorocitric acid, inhibition of soluble aconitase by (Peters, R. A.) 11P
Formaldehyde, reaction of tetrahydropteroylglutamic acid and related hydropteridines with (Blakley, R. L.) 707
Formate, utilization of, by cell-free extracts of *Pseudomonas oxalaticus* (OX 1) (Quayle, J. R. & Keech, D. B.) 631
Formate, utilization of, by *Pseudomonas oxalaticus* (OX 1) (Quayle, J. R. & Keech, D. B.) 623
Formic dehydrogenase, presence of, in *Pseudomonas oxalaticus* (OX 1) (Quayle, J. R. & Keech, D. B.) 631
Fructose diphosphate, effect of, on incorporation of amino acids into microsomes (Rendi, R. & Campbell, P. N.) 34
6^G-Fructosylsucrose in banana (Henderson, R. W., Morton, R. K. & Rawlinson, W. A.) 340
Fungicides, activity of griseofulvin relatives as (Crowdy, S. H., Grove, J. F. & McCloskey, P.) 241

Glucosaccharo-1→4-lactone, inhibition of β-glucuronidase by (Boyland, E., Davies, J. H. & Williams, K.) 28P
[^{14}C]Glucose, incorporation of, by *Escherichia coli* (Meadow, P. & Work, E.) 400
Glucose 6-phosphate dehydrogenase, adaptation of, to growth of *Escherichia coli* in complex media (Scott, D. B. McNair & Chu, E.) 426
Glucose, production of pyruvic acid, oxaloacetic acid and α-oxoglutaric acid from, by tissue in culture (Abdel-Tawab, G. A., Broda, E. & Kellner, G.) 619
Glucose, studies on the conversion of, into lactic acid in the Ehrlich ascites tumour (Dewey, D. L. & Green, F. O.) 160
β-Glucuronidase, inhibition of, by glucosaccharo-1→4-lactone (Boyland, E., Davies, J. H. & Williams, K.) 28P
γ-Glutamyl peptides, metabolism of, in subcellular fractions of rabbit liver (Cliffe, E. E. & Waley, S. G.) 12P
Glutathione, biosynthesis of, by rat erythrocytes (Kasbekar, D. K. & Sreenivasan, A.) 389
Glutathione disulphide, effect of vitamin B_{12} on reduction of, in human erythrocytes (Jocelyn, P. C.) 11P
Glutathione reductase, enzymic studies of X-irradiated cornea and lens with special reference to (Kuhlman, R. E. & Resnik, R. A.) 261
Glycerol, metabolism of, in *Mycobacterium butyricum* (Edson, N. L., Hunter, G. J. E., Kulka, R. G. & Wright, D. E.) 249
Glycogens isolated from livers of foetal animals, molecular structure of (Warren, F. L. & Whittaker, M.) 288
Glycolysis associated with brain mitochondria, point of aerobic inhibition of (Balázs, R.) 561
Glycolysis in pea-seed extracts, aerobic inhibition of, and its relationship to the Pasteur effect (Hatch, M. D. & Turner, J. F.) 524
Glycolysis in rat retina, effect of an inherited retinal degeneration on development of (Graymore, C. N., Tansley, K. & Kerly, M.) 459
Glyoxylate cycle, assay methods for key enzymes of (Dixon, G. H. & Kornberg, H. L.) 3P
Glyoxylate cycle enzymes, influence of growth substrates on levels of, in *Pseudomonas ovalis* (Kornberg, H. L. & Lund, P.) 33P
Glyoxylate plus oxaloacetate as inhibitor of aconitase (Ruffo, A., Romano, M. & Adinolfi, A.) 613
Goat, glycogen from foetus of (Warren, F. L. & Whittaker, M.) 288
Goldfish muscles, acetylcholinesterase in (Lundin, S. J.) 210
Griseofulvin relatives in plant tissue, estimation of (Crowdy, S. H., Green, A. P., Grove, J. F., McCloskey, P. & Morrison, A.) 230
Griseofulvin relatives, systemic fungicidal activity and chemical structure in (Crowdy, S. H., Grove, J. F. & McCloskey, P.) 241
Growth hormone, purification of, from pig pituitary gland (Ottaway, J. H.) 22P
Guanidinoacetic acid and other substituted guanidines, occurrence of, in mammalian liver (Rosenberg, H.) 582
γ-Guanidinobutyric acid, detection of, in rat liver (Rosenberg, H.) 582
β-Guanidinopropionic acid, detection of, in rat liver (Rosenberg, H.) 582

Haemorrhage and tissue electrolytes (Widdowson, E. M. & Southgate, D. A. T.) 200
Haptoglobins, human, estimation and purification of (Connell, G. E. & Smithies, O.) 115
Hartnup disease, metabolic abnormality of (Milne, M. D., Crawford, M. A., Girão, C. B. & Loughridge, L.) 30P
Heart, oxidation–reduction potential of cytochrome *b* in preparation from muscle of (Colpa-Boonstra, J. & Holton, F. A.) 4P
Heart, rate of reduction of endogenous ubiquinone in preparation from muscle of (Pumphrey, A. M. & Redfearn, E. R.) 2P
Hemlock (*Conium maculatum* L.), alkaloids of (Fairbairn, J. W. & Challen, S. B.) 556
Hevea brasiliensis latex, ribonucleoprotein component of (McMullen, A. I.) 545
cyclo[^{14}C]Hexane and its derivatives, metabolism of (Elliott, T. H., Parke, D. V. & Williams, R. T.) 193
Hexoestrol, tritium-labelled, selective accumulation of, by reproductive organs of immature female goats and sheep (Glascock, R. F. & Hoekstra, W. G.) 673
Histones, thymus, chromatography of (Phillips, D. M. P. & Johns, E. W.) 538
Hyaluronic acid complex of synovial fluid, degradation of, by proteolytic enzymes and by ethylenediaminetetra-acetic acid (Ogston, A. G. & Sherman, T. F.) 301
§Hydrolytic enzymes, steric factors in action of (Webb, E. C.) 41P
Hydroxamic acids, reactivation of phosphorylated chymotrypsin by (Green, A. L. & Nicholls, J. D.) 70
6-Hydroxy-2-methylaminopurine, presence of, in ribonucleic acids (Smith, J. D. & Dunn, D. B.) 294
21-Hydroxy-5α-pregnane-3:20-dione and its 5β-isomer as metabolites of deoxycorticosterone (Taylor, W.) 442
Hydroxypyruvate in animal tissues, metabolism of (Dickens, F. & Williamson, D. H.) 496
Hydroxystilbenes, occurrence of, in *Eucalyptus* (Hathway, D. E. & Seakins, J. W. T.) 37P
Hydroxystilbenes of *Eucalyptus wandoo* (Hathway, D. E. & Seakins, J. W. T.) 369
Hypoglycaemic coma, irreversible, in sheep after injections of insulin (Setchell, B. P.) 275

*myo*Inositol 2-phosphate, a constituent of liver monophosphoinositide (Hawthorne, J. N. & Hübscher, G.) 10P
Insulin, irreversible hypoglycaemic coma in sheep after injections of (Setchell, B. P.) 275
Intestine, sterols in cell fractions from mucosa of, in fasted rats and rats dosed with 7-dehydrocholesterol (Glover, J. & Stainer, D. W.) 79
Intestine, uptake of sterols by organelles in mucosa of (Glover, J., Green, C. & Stainer, D. W.) 82
Invertase, oligosaccharide synthesis in the banana and its relationship to the transferase activity of (Henderson, R. W., Morton, R. K. & Rawlinson, W. A.) 340
Iodoacetate, hypersensitivity of the visual cell to (Graymore, C. N.) 23P
o-Iodophenols, reversible photosensitive changes on alkalinification of solutions of, in aqueous organic solvents (Tata, J. R.) 214
Ion transport and metabolism in slices of guinea-pig seminal-vesicle mucosa (Whittam, R. & Breuer, H. J.) 638
Iron, stimulation of liver acid phosphatase activity after the parenteral injection of excessive doses of (Golberg, L., Martin, L. E., Bates, C. M. & Batchelor, A.) 20P
Isoniazid, effect of, on growth and transaminases of *Escherichia coli* (Hicks, R. M.) 22P

Ketone bodies, renal threshold for (Stewart, H. B. & Young, F. G.) 60
Ketonuria, stimulation of, by a substance from animal tissues (Stewart, H. B. & Young, F. G.) 60
Ketosis, bovine, biochemical aspects of (Bach, S. J. & Hibbitt, K. G.) 87
Kidney, human, normal and diseased, unsaponifiable fraction of lipid from (Mervyn, L. & Morton, R. A.) 106
Kidney, metabolism of thyroid-gland hormone by (Galton, V. A. & Pitt-Rivers, R.) 314
Kidney, sheep, enzyme systems of (Gallagher, C. H. & Buttery, S. H.) 575

Lactic acid, studies on the conversion of glucose into, in the Ehrlich ascites tumour (Dewey, D. L. & Green, F. O.) 160
Lactic dehydrogenase, human-liver, demonstration of auto-antibodies reacting with (Burton, P., Kerr, L. M. H. & Frazer, S. C.) 12P
Lactobacillus bifidus, dextranase of (Bailey, R. W. & Clarke R. T. J.) 49
Lactobacillus brevis, pyruvate metabolism in (Walker, J. R. L.) 188
Latex (*Hevea brasiliensis*), ribonucleoprotein component of (McMullen, A. I.) 545
Lathosterol, formation of, from 7-dehydrocholesterol in rat intestinal mucosa (Glover, J. & Stainer, D. W.) 79
Lecithin, activation of surface films of, by amphipathic molecules (Dawson, R. M. C. & Bangham, A. D.) 493
Lecithinase, relation between activity of, and the electrophoretic charge of the substrate (Bangham, A. D. & Dawson, R. M. C.) 486
Lens, cattle, distribution of sodium and potassium within (Amoore, J. E., Bartley, W. & van Heyningen, R.) 126
Lens, X-irradiated, enzymic studies of, with special reference to glutathione reductase (Kuhlman, R. E. & Resnik, R. A.) 261
Leucine, incorporation of, into ribonucleic acid from liver tumour (Greengard, O. & Campbell, P. N.) 305
Lipid, unsaponifiable fraction of, from normal and diseased human kidney (Mervyn, L. & Morton, R. A.) 106
Lipophosphoprotein complex in hen plasma associated with yolk production (McIndoe, W. M.) 153
Liver cells, acute toxic action of dimethylnitrosamine on (Bailie, M. J. & Christie, G. S.) 473
Liver, duck, incorporation of amino acids into proteins of isolated microsome fraction from (Campbell, P. N. & Kernot, B. A.) 6P
Liver, foetal, molecular structure of glycogens isolated from (Warren, F. L. & Whittaker, M.) 288
Liver homogenate, investigation of products of metabolism after incubation of deoxycorticosterone with (Taylor, W.) 442
Liver, human, lactic dehydrogenase of, demonstration of autoantibodies reacting with (Burton, P., Kerr, L. M. H. & Frazer, S. C.) 12P
Liver, *myo*inositol 2-phosphate in monophosphoinositide of (Hawthorne, J. N. & Hübscher, G.) 10P
Liver, mammalian, occurrence of guanidinoacetic acid and other substituted guanidines in (Rosenberg, H.) 582
Liver, normal and carcinomatous, incorporation of amino acid into protein of microsome and mitochondria preparations from (Greengard, O. & Campbell, P. N.) 305
Liver, ox, isolation of phosphatidic acid from (Hübscher, G. & Clark, B.) 7P
Liver, rabbit, metabolism of γ-glutamyl peptides in subcellular fractions of (Cliffe, E. E. & Waley, S. G.) 12P
Liver, rat, anaerobic metabolism of acetaldehyde in suspensions of (Lundquist, F., Fugmann, U., Kläning, E. & Rasmussen, H.) 409
Liver, rat, effects of salicylate on mitochondria from (Jeffrey, S. W. & Smith, M. J. H.) 462
Liver, rat, vitamin B_{12} protection in experimental injury to, by carbon tetrachloride (Kasbekar, D. K., Lavate, W. V., Rege, D. V. & Sreenivasan, A.) 384

Liver, separation by countercurrent distribution of dinitrophenylated and methylated phospholipids from (Collins, F. D.) 281
Liver, sheep, enzyme systems of (Gallagher, C. H. & Buttery, S. H.) 575
Liver, unsaponifiable constituents of, ubiquinone and substance SC in various species (Cunningham, N. F. & Morton, R. A.) 92
Lymphatic tissues, rat, normal and neoplastic, incorporation of amino acid into cell-free preparations of (Rendi, R. & Campbell, P. N.) 34
[^{14}C]Lysine, incorporation of, by *Escherichia coli* (Meadow, P. & Work, E.) 400
Lysine, incorporation of, into ribonucleic acid from liver tumour (Greengard, O. & Campbell, P. N.) 305

Macrophages, ribonucleic acid turnover in (Watts, J. W. & Harris, H.) 147
Mammary gland, guinea-pig, properties of mitochondria from (Jones, E. A. & Gutfreund, H.) 31P
Mammary gland, guinea-pig, reactions of amino acids with soluble ribonucleic acid from cells of (Fraser, M. J., Shimizu, H. & Gutfreund, H.) 141
Methionine, relationship between cobalamin and synthesis of, in *Escherichia coli* (Guest, J. R.) 5P
2-Methoxynaphthalene, metabolism of, by *Aspergillus niger* (Byrde, R. J. W., Downing, D. F. & Woodcock, D.) 344
1-Methylguanine, presence of, in ribonucleic acids (Smith, J. D. & Dunn, D. B.) 294
Mevalonic acid, formation of 'terpenoid' acids from, in liver enzyme preparations and their relation to sterol biosynthesis (Popják, G., Horning, M., Bucher, N. L. R. & Cornforth, R. H.) 34P
Mevalonic kinase in liver (Levy, H. R. & Popják, G.) 35P
Michaelis constant for alkaline phosphatases, factors influencing (Motzok, I. & Branion, H. D.) 177
Micrococcus lysodeikticus, products of partial acid hydrolysis of mucopeptide from cell walls of (Perkins, H. R. & Rogers, H. J.) 647
Micrococcus lysodeikticus, succinyl-group translocation through the plasma membrane of (Mitchell, P. & Moyle, J.) 21P
Microsome fraction, duck-liver, isolated, incorporation of amino acids into proteins of (Campbell, P. N. & Kernot, B. A.) 6P
Microsomes, incorporation of amino acid into protein of preparations of, from liver and liver tumour (Greengard, O. & Campbell, P. N.) 305
Microsomes, role of cytoplasmic ribonucleic acid in incorporation of amino acids into proteins of (Rendi, R. & Campbell, P. N.) 435
Micro-tests for sugars (Clarke, E. G. C.) 13P
Milk, human, fatty acids of, from mothers on diets taken *ad libitum* (Insull, W., Jun. & Ahrens, E. H., Jun.) 27
Mitochondria, brain, point of aerobic inhibition of glycolysis associated with (Balázs, R.) 561
Mitochondria, incorporation of amino acid into protein of preparations of, from liver and liver tumour (Greengard, O. & Campbell, P. N.) 305
Mitochondria, mammary gland, guinea-pig, properties of (Jones, E. A. & Gutfreund, H.) 31P
Mitochondria, rat-liver, effects of salicylate on (Jeffrey, S. W. & Smith, M. J. H.) 462
Mitochondria, rat-liver, isolated, incorporation of amino acid into proteins of (Reis, P. J., Coote, J. L. & Work, T. S.) 24P
Monoiodoacetate, effect of, on aerobic metabolism of ox retina *in vitro* (Hopkinson, L. & Kerly, M.) 22
Monophosphoinositide, liver, *myo*inositol 2-phosphate a constituent of (Hawthorne, J. N. & Hübscher, G.) 10P
Mosquito, studies on the adenosine triphosphate–phosphate exchange and the hydrolysis of adenosine triphosphate catalysed by a particulate fraction from (Avi-Dor, Y. & Gonda, O.) 8
Mucin, cervical, bovine, physical and chemical properties of mucoids from (Gibbons, R. A.) 27P
Mucoids from bovine cervical mucin, physical and chemical properties of (Gibbons, R. A.) 27P
Mucopeptide from cell walls of *Micrococcus lysodeikticus*, products of partial acid hydrolysis of (Perkins, H. R. & Rogers, H. J.) 647
Muscle, goldfish, acetylcholinesterase in (Lundin, S. J.) 210
Muscle, heart, oxidation–reduction potential of cytochrome *b* in preparation from (Colpa-Boonstra, J. & Holton, F. A.) 4P
Muscle, heart, rate of reduction of endogenous ubiquinone in preparation from (Pumphrey, A. M. & Redfearn, E. R.) 2P
Muscle, pectoral, changes in erythrocyte chemistry accompanying temporary lesions in, in young fowls (Bell, D. J. & Siller, W. G.) 27P
Muscle, skeletal, ribonucleoprotein of, and its relation to the myofibril (Perry, S. V. & Zydowo, M.) 682
Mycobacterium butyricum, metabolism of glycerol in (Edson, N. L., Hunter, G. J. E., Kulka, R. G. & Wright, D. E.) 249
Myleran (1:4-dimethanesulphonyloxybutane), studies on the metabolism of (Roberts, J. J. & Warwick, G. P.) 3P
Myofibril, ribonucleoprotein of skeletal muscle and its relation to (Perry, S. V. & Zydowo, M.) 682
L-Myosin, effects of adenosine triphosphate on mechanical properties of surface films of (Cheesman, D. F., Keeler, M. M. & Sten-Knudsen, O.) 357
Myrica, observations on composition and metabolism of nitrogen-fixing root nodules of (Leaf, G., Gardner, I. C. & Bond, G.) 662

Nicotinic acid hydroxylase system of *Pseudomonas fluorescens* KB 1, purification of (Hunt, A. L.) 1
Ninhydrin-reacting substances, two-way separation of amino acids and, by high-voltage electrophoresis followed by paper chromatography (Efron, M. L.) 691
Ninhydrin reaction, low-temperature, estimation of anserinase activity by (Jones, N. R.) 407
Nitrogen-fixing root nodules of *Myrica*, observations on composition and metabolism of (Leaf, G., Gardner, I. C. & Bond, G.) 662
Nitro-reductase enzyme, purification of, of a *Nocardia* sp. (Villanueva, J. R.) 36P
Nocardia sp., purification of nitro-reductase enzyme of (Villanueva, J. R.) 36P
Nuclei, effect of ionizing radiation on phosphorylation by, of radio-sensitive tissues of rat (Creasey, W. A. & Stocken, L. A.) 519
Nucleic acids, turnover of, in rabbit macrophages (Watts, J. W. & Harris, H.) 147
Nucleolus, function of, and initiation of deoxyribonucleic acid synthesis in the connective-tissue cell (Harris, H.) 54
Nucleoside diphosphokinase of pea seeds (Kirkland, R. J. A. & Turner, J. F.) 716
§Nucleotide metabolism, stereochemical specificities of enzymes of (Barker, G. R.) 42P
Nucleotides in banana (Henderson, R. W., Morton, R. K. & Rawlinson, W. A.) 340

Oligosaccharide synthesis in the banana and its relationship to the transferase activity of invertase (Henderson, R. W., Morton, R. K. & Rawlinson, W. A.) 340
Ornithuric acid formation in hens (Baldwin, B. C., Robinson, D. & Williams, R. T.) 35P
Oxaloacetate plus glyoxylate as inhibitor of aconitase (Ruffo, A., Romano, M. & Adinolfi, A.) 613

Oxaloacetic acid, excretion of, by *Mycobacterium butyricum* (Edson, N. L., Hunter, G. J. E., Kulka, R. G. & Wright, D. E.) 249
Oxaloacetic acid, production of, from glucose by tissue in culture (Abdel-Tawab, G. A., Broda, E. & Kellner, G.) 619
§Oxidation–reduction enzymes, steric factors in action of (Slater, E. C.) 41P
Oxidation–reduction potential of cytochrome *b* in heart-muscle preparation (Colpa-Boonstra, J. & Holton, F. A.) 4P
Oximes, reactivation of phosphorylated chymotrypsin by (Green, A. L. & Nicholls, J. D.) 70
α-Oxoglutaric acid, production of, from glucose by tissue in culture (Abdel-Tawab, G. A., Broda, E. & Kellner, G.) 619

Pancreas, study of 'unsaponifiable' extracts obtained from hydrolysates of (Morton, R. A. & Fahmy, N. I.) 99
Pasteur effect, aerobic inhibition of glycolysis in pea-seed extracts and its relationship to (Hatch, M. D. & Turner, J. F.) 524
Pea seeds, nucleoside diphosphokinase of (Kirkland, R. J. A. & Turner, J. F.) 716
Pea-seed extracts, aerobic inhibition of glycolysis in, and its relationship to the Pasteur effect (Hatch, M. D. & Turner, J. F.) 524
Penicillium spinulosum, component fatty acids of fat from (Shimi, I. R., Singh, J. & Walker, T. K.) 184
Pentamidine, effect of, on growth and transaminases of *Escherichia coli* (Hicks, R. M.) 22P
pH optima for alkaline phosphatases, factors influencing (Motzok, I. & Branion, H. D.) 177
Phosphatase activity, acid, liver, stimulation of, after the parenteral injection of excessive doses of iron (Golberg, L., Martin, L. E., Bates, C. M. & Batchelor, A.) 20P
Phosphatase, plasma, kinetics of, of normal and rachitic chicks (Motzok, I.) 169
Phosphatases, alkaline, factors influencing pH optima and Michaelis constant for (Motzok, I. & Branion, H. D.) 177
Phosphatidic acid, isolation of, from ox liver (Hübscher, G. & Clark, B.) 7P
6-Phosphogluconate dehydrogenase in *Escherichia coli* (Scott, D. B. McNair & Chu, E.). 426
Phospholipid, complex, from sheep brain (Collins, F. D.) 532
Phospholipid fractions from butter (Billimoria, J. D. & Curtis, R. G.) 29P
Phospholipids, dinitrophenylated and methylated, separation of, by countercurrent distribution (Collins, F. D.) 281
Pituitary gland, pig, purification of growth hormone from (Ottaway, J. H.) 22P
Plasma, hen, lipophosphoprotein complex in, associated with yolk production (McIndoe, W. M.) 153
Plum, incorporation of ^{14}C into the sorbitol of leaves of (Anderson, J. D., Andrews, P. & Hough, L.) 9P
Polyacetylenes, biosynthesis of, by a basidiomycete (Bu'Lock, J. D. & Gregory, H.) 322
Porphobilinogen, biosynthesis of porphyrins from, by *Rhodopseudomonas spheroides* (Heath, H. & Hoare, D. S.) 14
Porphyrins, biosynthesis of, from porphobilinogen by *Rhodopseudomonas spheroides* (Heath, H. & Hoare, D. S.) 14
Potassium, distribution of, within cattle lens (Amoore, J. E., Bartley, W. & van Heyningen, R.) 126
Propamidine, effect of, on growth and transaminases of *Escherichia coli* (Hicks, R. M.) 22P
Protein antigens, intravenously injected, recovery of, from rat spleen (Hawkins, J. D. & Haurowitz, F.) 5P
Protein, location of ^{14}C in, from isolated rat diaphragm incubated with [^{14}C]amino acids and with $^{14}CO_2$ (Manchester, K. L. & Young, F. G.) 136
Proteinase content of thymus histones (Phillips, D. M. P. & Johns, E. W.) 538
Proteinase, purification and properties of, from *Bacillus cereus* (Salter, D. N.) 23P
Protein-binding, study of, in metabolism of vitamin B_{12} (Gregory, M. E. & Holdsworth, E. S.) 549
Proteins, incorporation of amino acids into, of isolated microsome fraction from duck liver (Campbell, P. N. & Kernot, B. A.) 6P
Proteins, interaction between thyroxine and (Tata, J. R.) 222
Proteins, microsomal, role of cytoplasmic ribonucleic acid in incorporation of amino acids into (Rendi, R. & Campbell, P. N.) 435
Proteins of isolated rat-liver mitochondria, incorporation of amino acid into (Reis, P. J., Coote, J. L. & Work, T. S.) 24P
Proteins, polarization of ultraviolet fluorescence and electronic energy transfer in (Weber, G. & Teale, F. W. J.) 15P
Proteins, ultraviolet fluorescence of (Teale, F. W. J. & Weber, G.) 15P
Proteolysis, gastric, in diseases affecting the stomach (Taylor, W. H.) 1P
Pseudomonas fluorescens, purification of the nicotonic acid hydroxylase system of (Hunt, A. L.) 1
Pseudomonas ovalis, influence of growth substrates on levels of glyoxylate cycle enzymes in (Kornberg, H. L. & Lund, P.) 33P
Pseudomonas oxalaticus (OX 1), formate and carbon dioxide utilization by cell-free extracts of, grown on formate (Quayle, J. R. & Keech, D. B.) 631
Pseudomonas oxalaticus (OX 1), formate and carbon dioxide utilization by, during growth on formate (Quayle, J. R. & Keech, D. B.) 623
Purine and its derivatives, actions of some enzymes on (Barker, G. R., Lloyd, J. B., Montague, M. D. & Wood, N. F.) 9P
Purines, role of, in flavinogenesis in *Candida flareri* (Audley, B. G., Goodwin, T. W. & McEvoy, D.) 8P
Pyridoxal phosphate, interaction between amino acids and (King, H. K. & Lucas, N.) 18P
Pyruvate metabolism in *Lactobacillus brevis* (Walker, J. R. L.) 188
Pyruvic acid, production of, from glucose by tissue in culture (Abdel-Tawab, G. A., Broda, E. & Kellner, G.) 619

Rachitis, kinetics of plasma phosphatase of normal chicks and chicks with (Motzok, I.) 169
Radiation, ionizing, effect of, on nuclear phosphorylation in radio-sensitive tissues of rat (Creasey, W. A. & Stocken, L. A.) 519
Retina, ox, effect of monoiodoacetate on aerobic metabolism of, *in vitro* (Hopkinson, L. & Kerly, M.) 22
Retina, rat, effect of an inherited retinal degeneration on development of glycolysis in (Graymore, C. N., Tansley, K. & Kerly, M.) 459
§Retinene in visual processes, *cis–trans* isomers of (Pitt, G. A. J. & Morton, R. A.) 40P
Rhodopseudomonas spheroides, adaptation to form bacteriochlorophyll in (Lascelles, J.) 508
Rhodopseudomonas spheroides, biosynthesis of porphyrins from porphobilinogen by (Heath, H. & Hoare, D. S.) 14
Ribonuclease, effect of, on incorporation of amino acid into mitochondrial protein (Greengard, O. & Campbell, P. N.) 305

Ribonucleic acid, cytoplasmic, role of, in incorporation of amino acids into microsomal proteins (Rendi, R. & Campbell, P. N.) 435
Ribonucleic acid, soluble, from guinea-pig mammary cells, reactions of amino acids with (Fraser, M. J., Shimizu, H. & Gutfreund, H.) 141
Ribonucleic acids from several sources, occurrence of methylated guanines in (Smith, J. D. & Dunn, D. B.) 294
Ribonucleoprotein component of *Escherichia coli* (Bowen, T. J., Dagley, S. & Sykes, J.) 419
Ribonucleoprotein component of *Hevea brasiliensis* latex (McMullen, A. I.) 545
Ribonucleoprotein of skeletal muscle and its relation to the myofibril (Perry, S. V. & Zydowo, M.) 682
Ribonucleoprotein, rat-liver, exposed to cold trichloroacetic acid, effect of ethanol on (Venkataraman, P. R. & Lowe, C. U.) 430
Rumen cellulolytic enzymes, substrate specificity of (Festenstein, G. N.) 75

Salicylate, effects of, on mitochondria from rat liver (Jeffrey, S. W. & Smith, M. J. H.) 462
Sarcina lutea, patterns of oxidative assimilation in (Binnie, B., Holms, W. H. & Dawes, E. A.) 17P
Seminal-vesicle mucosa, guinea-pig, ion transport and metabolism in slices of (Whittam, R. & Breuer, H. J.) 638
L-Serine, metabolism of, in liver (Dickens, F. & Williamson, D. H.) 496
Serine transhydroxymethylase, preparation of (Blakley, R. L.) 707
Serum albumin, human, intermolecular and intramolecular reactions of, after X-irradiation in aqueous solution (Rosen, D.) 597
Sheep, irreversible hypoglycaemic coma in (Setchell, B. P.) 275
Sheep, normal, cerebral metabolism in (Setchell, B. P.) 265
Sheep tissues, biochemistry of (Gallagher, C. H. & Buttery, S. H.) 575
Sodium, distribution of, within cattle lens (Amoore, J. E., Bartley, W. & van Heyningen, R.) 126
Sorbitol of plum leaves, incorporation of ^{14}C into (Anderson, J. D., Andrews, P. & Hough, L.) 9P
Spinach leaves, photosynthetic uptake of [^{14}C]carbon dioxide by subcellular components of (Moses, V., Holm-Hansen, O., Pon, N. G., Nishida, K. & Calvin, M.) 19P
Spleen, rat, recovery of intravenously injected protein antigens from (Hawkins, J. D. & Haurowitz, F.) 5P
Spleen, study of 'unsaponifiable' extracts obtained from hydrolysates of (Morton, R. A. & Fahmy, N. I.) 99
Staphylococcus aureus, incorporation of amino acids into cell-wall mucopeptide of, and effect of antibiotics on the process (Mandelstam, J. & Rogers, H. J.) 654
§Stereochemical correlations (Klyne, W.) 39P
§Stereochemical specificities of enzymes of nucleotide metabolism (Barker, G. R.) 42P
§Steroids, steric aspects of biosynthesis of terpenes and (Arigoni, D.) 39P
Sterol biosynthesis, formation of 'terpenoid' acids from mevalonic acid in liver-enzyme preparations and their relation to (Popják, G., Horning, M., Bucher, N. L. R. & Cornforth, R. H.) 34P
Sterols, site of esterification of, during intestinal absorption (Glover, J., Green, C. & Stainer, D. W.) 82
Stomach, gastric proteolysis in diseases affecting (Taylor, W. H.) 1P
Streptococcus bovis, transglucosidase activity of rumen strains of (Bailey, R. W.) 42
Substance SC, presence of, in liver of various species (Cunningham, N. F. & Morton, R. A.) 92
Succinyl group, translocation of, through the plasma membrane of *Micrococcus lysodeikticus* (Mitchell, P. & Moyle, J.) 21P
Sugars, micro-tests for (Clarke, E. G. C.) 13P
Sulphate, enzymically liberated, use of barium chloranilate in the determination of (Lloyd, A. G.) 133
Swayback, copper content and cytochrome oxidase activity of tissues from lambs with (Howell, J. McC. & Davison, A. N.) 365
Synovial fluid, degradation of the hyaluronic acid complex of, by proteolytic enzymes and by ethylenediaminetetraacetic acid (Ogston, A. G. & Sherman, T. F.) 301

Tenuazonic acid, structure of (Stickings, C. E.) 332
§Terpenes, steric aspects of biosynthesis of steroids and (Arigoni, D.) 39P
'Terpenoid' acids, formation of, from mevalonic acid in liver enzyme preparations and their relation to sterol biosynthesis (Popják, G., Horning, M., Bucher, N. L. R. & Cornforth, R. H.) 34P
Tetrahydropteroylglutamic acid and related hydropteridines, reaction of, with formaldehyde (Blakley, R. L.) 707
Tetraiodothyroacetic acid, identification of, as a metabolite in mouse kidney (Galton, V. A. & Pitt-Rivers, R.) 319
Thetin–homocysteine transmethylase, distribution of, studied with the aid of trimethylsulphonium chloride as substrate (Maw, G. A.) 602
Thiols, blood, chromatographic analysis of (Smith, M. J. & Rodnight, R.) 1P
Thymus, chromatography of histones from (Phillips, D. M. P. & Johns, E. W.) 538
Thyroid-gland hormone, metabolism of, in kidney (Galton, V. A. & Pitt-Rivers, R.) 314
Thyroid-gland hormones, separation of, from serum and tissues on column of Dowex-1 resin (Galton, V. A. & Pitt-Rivers, R.) 310
Thyrotoxicosis, experimental, rat, vitamin B_{12} protection in (Kasbekar, D. K., Lavate, W. V., Rege, D. V. & Sreenivasan, A.) 374
Thyroxine, binding of, by human- and chicken-serum proteins (Tata, J. R. & Shellabarger, C. J.) 608
Thyroxine, biological half-life of, in chickens (Tata, J. R. & Shellabarger, C. J.) 608
Thyroxine, identification of acetic acid analogues of, in mammalian tissues (Galton, V. A. & Pitt-Rivers, R.) 319
Thyroxine, interaction between proteins and (Tata, J. R.) 222
Thyroxine, reversible photosensitive changes on alkalinification of solutions of, in aqueous organic solvents (Tata, J. R.) 214
Thyroxine, ultraviolet-absorption spectrum of (Tata, J. R.) 214
Tissue, apparatus for incubating slices of, *in vitro* (Ottaway, J. H., Huggins, A. K. & Martin, J.) 25P
Transaminases of *Escherichia coli*, effect of isoniazid, propamidine, pentamidine and carbutamide on (Hicks, R. M.) 22P
Transferase activity of invertase, oligosaccharide synthesis in the banana and its relationship to (Henderson, R. W., Morton, R. K. & Rawlinson, W. A.) 340
Transferrins, genetic control of, in humans (Smithies, O. & Hiller, O. 121
Transglucosidase activity of rumen strains of *Streptococcus bovis* (Bailey, R. W.) 42
1:1:1-Trichloro-2:2-di-(*p*-chlorophenyl)ethane, effect of, on adenosine triphosphate–phosphate exchange (Avi-Dor, Y. & Gonda, O.) 8
2:4:5-Trihydroxybutyrophenone, metabolism of, in rat and dog (Astill, B. D., Fassett, D. W. & Roudabush, R. L.) 451
3:5:4′-Trihydroxystilbene in heartwood of *Eucalyptus wandoo* (Hathway, D. E. & Seakins, J. W. T.) 369
Tri-iodothyroacetic acid, identification of, as a metabolite in mouse liver (Galton, V. A. & Pitt-Rivers, R.) 319

Tri-iodothyronine, biological half-life of, in chickens (Tata, J. R. & Shellabarger, C. J.) 608
3:5:3′-Tri-iodothyronine, reversible photosensitive changes on alkalinification of solutions of, in aqueous organic solvents (Tata, J. R.) 214
3:5:3′-Tri-iodothyronine, ultraviolet-absorption spectrum of (Tata, J. R.) 214
Trimethylsulphonium chloride as substrate for thetin–homocysteine transmethylase (Maw, G. A.) 602
Tryptophan, 1-(*o*-carboxyphenylamino)-1-deoxyribulose as intermediate in biosynthesis of, by mutant strains of *Aerobacter aerogenes* and *Escherichia coli* (Doy, C. H. & Gibson, F.) 586
Tumour, Ehrlich ascites, studies on conversion of glucose into lactic acid in (Dewey, D. L. & Green, F. O.) 160
Tumour, liver, incorporation of amino acid into protein of microsome and mitochondria preparations of (Greengard, O. & Campbell, P. N.) 305

Ubiquinone, endogenous, rate of reduction of, in heart-muscle preparation (Pumphrey, A. M. & Redfearn, E. R.) 2P
Ubiquinone, presence of, in liver of various species (Cunningham, N. F. & Morton, R. A.) 92
Urea, role of, in flavinogenesis in *Candida flareri* (Audley, B. G., Goodwin, T. W. & McEvoy, D.) 8P

Vicia faba see Bean
Vitamin A, intestinal absorption of, in the guinea pig (Glover, J., Green, C. & Stainer, D. W.) 82
Vitamin B_{12}, effect of, on reduction of glutathione disulphide in human erythrocytes (Jocelyn, P. C.) 11P
Vitamin B_{12} protection in experimental liver injury to the rat by carbon tetrachloride (Kasbekar, D. K., Lavate, W. V., Rege, D. V. & Sreenivasan, A.) 384
Vitamin B_{12} protection in experimental thyrotoxicosis in the rat (Kasbekar, D. K., Lavate, W. V., Rege, D. V. & Sreenivasan, A.) 374
Vitamin B_{12}, study of protein-binding in metabolism of (Gregory, M. E. & Holdsworth, E. S.) 549
Vitamin D, kinetics of plasma phosphatase of normal chicks and chicks deficient in (Motzok, I.) 169

Yolk, lipophosphoprotein complex in hen plasma associated with production of (McIndoe, W. M.) 153

The 38
Road,

Gastr
B

Under
human
with tw
3·3–4·(
but no
two m
3·1–3·2

In p
abnorm
are so
with
normal
resemb
patient
pyloric
patient
increas
gastric
chronic
1958) c
'pseud

In p
patient
proteol
inactiv
pyloric
exhibit
five of
occurre

Chron
B

Prelim
for blo
steps,
oxidat
(*a*) ext
from n
by col
graphy

A pr
and te
cystein
satura

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society.*)

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **381st Meeting** *of the Biochemical Society was held in the Department of Biochemistry, South Parks Road, Oxford, on Friday, 20 March 1959, when the following papers were read:*

COMMUNICATIONS

Gastric Proteolysis in Diseases Affecting the Stomach. By W. H. TAYLOR. (*Department of Clinical Biochemistry, Radcliffe Infirmary, Oxford*)

Under defined conditions (Taylor, 1959) normal human gastric juice digests most protein substrates with two pH maxima in the pH ranges 1·7–2·4 and 3·3–4·0. Fundic mucosal extracts behave similarly, but normal human pyloric mucosal extracts exhibit two maxima at lower ranges of pH 1·5–1·6 and 3·1–3·2.

In patients with disease of the gastric mucosa, abnormal gastric proteolytic pH-activity curves are sometimes seen. Only three of ten patients with chronic hypochromic anaemia exhibited normal curves below pH 5; in five, the curves resembled those of pyloric extracts. Four of five patients with idiopathic steatorrhoea also exhibited pyloric-type curves. It is concluded that some patients with these diseases secrete a relatively increased quantity of pyloric-type pepsin in their gastric juice. There is histological evidence in chronic hypochromic anaemia (Lees & Rosenthal, 1958) of a replacement of normal fundic glands by 'pseudo-pyloric' glands.

In pernicious anaemia, only one of twenty-three patients secreted gastric juice exhibiting a normal proteolytic pH-activity curve. Three secreted inactive juices, four secreted juices showing a pyloric-type curve and thirteen secreted juices exhibiting only a single maximum below pH 5. In five of these thirteen patients the single maximum occurred above pH 4 and thus at a point where normal gastric proteolytic activity declines rapidly. The abnormal pH-activity curves are tentatively explained as follows: (*a*) in those patients who show no activity, the fundic and pyloric glands are atrophic and/or non-secreting, (*b*) in those who show the pyloric-type of curve, the pyloric mucous membrane is preserved, (*c*) in those who show abnormal maxima, abnormal enzymes are elaborated and secreted.

The gastric proteolytic pH-activity curves of patients with peptic ulcer also differ from those of normal subjects. Three maxima below pH 5, at pH 1·5–2·2, 2·5–3·2 and 3·3–3·9, were exhibited by the histamine-stimulated gastric juices of seven of eleven patients, by the fundic mucosal extracts of twelve of sixteen patients and by the pyloric mucosal extracts of three of nine patients. No differences between patients with gastric and duodenal ulcer were detected. Present evidence suggests that these curves, too, result from the formation and secretion of an abnormal enzyme by the mucous membrane.

REFERENCES

Lees, R. & Rosenthal, F. D. (1958). *Quart. J. Med.* (N.S.), **27**, 19.

Taylor, W. H. (1959). *Biochem. J.* **71**, 73; and also D.M. Thesis, University of Oxford.

Chromatographic Analysis of Blood Thiols. By M. J. SMITH and R. RODNIGHT. (*Department of Biochemistry, Institute of Psychiatry, Maudsley Hospital, Denmark Hill, S.E.* 5)

Preliminary work on a chromatographic method for blood thiols indicated that the following four steps, carried out under conditions preventing the oxidation of the sulphydryl group, were required: (*a*) extraction from blood proteins; (*b*) separation from non-sulphydryl compounds; (*c*) fractionation by column chromatography; (*d*) paper chromatography.

A procedure for (*b*), (*c*) and (*d*) has been devised and tested with mixtures of glutathione (GSH), cysteinylglycine and cysteine. Dowex-2 resin saturated with *p*-chloromercuribenzoic acid (Besch, Goldzeiher & McCormack, 1957) was used to absorb the thiols and dimercaptopropanol (BAL) to elute them. Excess BAL was removed by ethyl acetate and immediately afterwards the sulphydryl group stabilized with *N*-ethylmaleimide (Hanes, Hird & Isherwood, 1950). The thiol-maleimide complexes were then fractionated on columns of Amberlite CG-120 operated with dilute HCl, and the fractions examined by paper chromatography.

Step (*a*) has proved difficult since many extracting agents encourage oxidation of thiols and others leave ions in the protein-free extract which inter-

fere with step (*b*). However, heat precipitation of blood proteins (Hunter, 1957), using CO-saturated blood (Numata, 1940), was found to give 95–100% recovery of GSH and an extract free of extraneous ions. When the thiol separation procedure was applied to heat-extracted human blood the following thiols were detected (concentrations in μg./ml. blood): GSH (200–300); cysteine (15–25); cysteinylglycine (0·5–2); thiols present at less than 0·5 μg./ml. would not have been detected. The identity of all three compounds was confirmed by chromatography in different solvents and by hydrolysis experiments. Recovery of thiols added to blood ranged from 60 to 90%. The GSH value (corrected for losses) agreed well with determinations by an amperometric method on unfractionated extracts; cysteine values agree with those quoted for cystine by Stein & Moore (1954). Present evidence suggests that at least part of the cysteinylglycine arises by hydrolysis of GSH during heat precipitation of the blood proteins; an alternative extraction method is therefore being sought. The results, however, do not support the work of Binkley, Fujii & Kimmel (1950), who reported the presence of much higher amounts of cysteinylglycine in blood.

We thank Professor H. McIlwain for his advice, Mr N. A. Gregson for help in the latter part of this work, and the William Shepherd Trust and the Research Fund of the Bethlem Royal and Maudsley Hospitals for a grant.

REFERENCES

Besch, P. K., Goldzeiher, J. W. & McCormack, S. (1957). *Science*, **126**, 650.
Binkley, F., Fujii, S. & Kimmel, J. R. (1950). *J. biol. Chem.* **186**, 159.
Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1950). *Nature, Lond.*, **166**, 288.
Hunter, G. (1957). *J. clin. Path.* **10**, 161.
Numata, I. (1940). *Biochem. Z.* **304**, 404.
Stein, W. H. & Moore, S. (1954). *J. biol. Chem.* **211**, 915.

The Rate of Reduction of the Endogenous Ubiquinone in a Heart-Muscle Preparation. By Alison M. Pumphrey and E. R. Redfearn. (*Department of Biochemistry, The University, Liverpool* 3)

Previous work has shown that ubiquinone is likely to be an active component of the respiratory chain (Crane, Hatefi, Lester & Widmer, 1957; Pumphrey, Redfearn & Morton, 1958*a*, *b*). In order to locate its site of action in the respiratory enzyme system the rates of reduction of endogenous ubiquinone by succinate and DPNH have been measured and the effects of various inhibitors on the reactions studied.

The enzyme preparation was obtained from pig heart by a modified Keilin & Hartree (1947) procedure, and it was found to have a ubiquinone concentration of 3·5–4·0 μmoles per g. protein. It showed succinate- and DPNH-ubiquinone reductase and ubiquinol oxidase activities. The activity of ubiquinol oxidase, however, appeared to depend on the type of buffer used as the suspending medium; with phosphate the activity was low, but it was greatly increased when the phosphate was replaced by tris.

In experiments with inhibitors it was found that amytal inhibited reduction of ubiquinone by DPNH and that cyanide inhibited oxidation of reduced ubiquinone, thus confirming the work of Hatefi, Lester & Ramasarma (1958). Reduction of ubiquinone by succinate was not inhibited by antimycin A (Kyowa) in a concentration which completely inhibited the succinic oxidase, but reoxidation of the ubiquinol was inhibited. These results are contrary to those of Hatefi *et al.* (1958), who reported that antimycin A inhibited ubiquinone reduction.

A method was developed for measuring the rates of reduction of endogenous ubiquinone by succinate and by DPNH. These rates were compared with those obtained from measurements of the succinic or DPNH oxidase activities made under similar conditions. From measurements made on a number of preparations it was found that the rate of succinate oxidation calculated from the ubiquinone reductase assay was 10–25% of that of the complete succinic oxidase system. Similar results were obtained using DPNH as substrate.

The results will be discussed in relation to the site of action of ubiquinone.

One of us (A.M.P.) is in receipt of a D.S.I.R. Studentship.

REFERENCES

Crane, F. L., Hatefi, Y., Lester, R. L. & Widmer, C. (1957). *Biochim. biophys. Acta*, **25**, 220.
Hatefi, Y., Lester, R. & Ramasarma, T. (1958). *Fed. Proc.* **17**, 238.
Keilin, D. & Hartree, E. F. (1947). *Biochem. J.* **41**, 500.
Pumphrey, A. M., Redfearn, E. R. & Morton, R. A. (1958*a*). *Chem. & Ind.* p. 978.
Pumphrey, A. M., Redfearn, E. R. & Morton, R. A. (1958*b*). *Biochem. J.* **70**, 1P.

Assay Methods for Key Enzymes of the Glyoxylate Cycle. By G. H. Dixon and H. L. Kornberg. (*M.R.C. Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford*)

The synthesis of cell constituents from acetate by bacteria, yeasts, fatty seedlings and fungi has been shown to involve the glyoxylate cycle (see Kornberg & Krebs, 1957; Kornberg & Madsen, 1958). Key enzymes of this cycle are *iso*citratase (Olson, 1954), catalysing the reaction

$$\textit{iso}\text{citrate} \rightleftharpoons \text{succinate} + \text{glyoxylate}, \quad \text{(i)}$$

and malate synthetase (Wong & Ajl, 1956), catalysing the reaction

$$\text{acetyl coenzyme A} + \text{glyoxylate} \rightarrow \text{malate} \quad \text{(ii)}$$

The following spectrophotometric assay procedures for these enzymes have been evolved.

(i) ***iso*Citratase.** The principle of this assay depends on measurements of the rate of increase of optical density at 324 mμ consequent upon the formation of glyoxylic acid phenylhydrazone from glyoxylate, produced by (i) from *iso*citrate, and phenylhydrazine. The assay system contains, in 3 ml., 200 μmoles of potassium phosphate, pH 6·85, 15 μmoles of $MgCl_2$, 10 μmoles of phenylhydrazine HCl, 6 μmoles of cysteine HCl, 0·05–1·0 ml. of biological extract to be assayed, and water. The reaction is started by the addition of 5 μmoles of potassium DL-*iso*citrate. After a lag of approx. 1 min., ΔE_{324} is linear for 4–5 min.; this rate is proportional to the enzyme concentration. ϵ_{324} for glyoxylic acid phenylhydrazone = $1{\cdot}7 \times 10^4$.

(ii) **Malate synthetase.** The principle of this assay depends on measurement of the rate of decrease of optical density at 232 mμ consequent upon breakage of the thio-ester bond of acetyl coenzyme A (Stadtman, 1957) in the presence of glyoxylate. The reaction system contains, in 0·42 ml., 18 μmoles of tris buffer, pH 7·1, 2 μmoles of $MgCl_2$, and 0·025 μmole of acetyl coenzyme A (Stadtman, 1957). Of the biological extract to be assayed, 0·005–0·02 ml. are added to the 'test' cell and the ΔE_{232} recorded for several minutes to detect the possible presence of an acetyl coenzyme A deacylase. Sodium glyoxylate (1 μmole) is then added; the initial rate of the rapid decrease in optical density is proportional to the amount of malate synthetase present. Condensing enzyme may be assayed in a similar manner by substituting oxaloacetate for glyoxylate in the above system. These assays demand use of spectrophotometers sensitive at high optical densities: a Cary recording spectrophotometer has been used in this work. The method has been checked by chemical estimation of malate (Hummel, 1949) and citrate (McArdle, 1955): the quantities formed in the reaction mixture correspond to the amounts of acetyl coenzyme A utilized. ϵ_{232} for the cleavage of the thio-ester bond of acetyl coenzyme A is $4{\cdot}5 \times 10^3$ (Stadtman, 1957). Results obtained by these methods will be presented.

REFERENCES

Hummel, J. P. (1949). *J. biol. Chem.* **180**, 1225.
Kornberg, H. L. & Krebs, H. A. (1957). *Nature, Lond.*, **179**, 988.
Kornberg, H. L. & Madsen, N. B. (1958). *Biochem. J.* **68**, 549.
McArdle, B. (1955). *Biochem. J.* **60**, 647.
Olson, J. A. (1954). *Nature, Lond.*, **174**, 695.
Stadtman, E. C. (1957). In *Methods in Enzymology*, vol. 3, p. 931. New York: Academic Press.
Wong, D. T. O. & Ajl, S. J. (1956). *J. Amer. chem. Soc.* **78**, 3220.

Studies on the Metabolism of Myleran (1:4-Dimethanesulphonyloxybutane). By J. J. Roberts and G. P. Warwick (introduced by L. A. Elson). (*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, S.W.* 3)

Earlier publications (Roberts & Warwick, 1957, 1958) described the presence of derivatives of *S*-alkylated cysteines in the urine of rats administered alkyl alkanesulphonates. Thus, for example, [^{14}C]ethyl methanesulphonate (I) was shown to be excreted as *N*-acetyl-*S*-ethylcysteine (II) and other derivatives of *S*-ethylcysteine.

$$CH_3SO_2OC_2H_5 \rightarrow C_2H_5{-}S{-}CH_2{-}\underset{\displaystyle NHCOCH_3}{\overset{\displaystyle |}{C}}HCOOH$$

(I) (II)

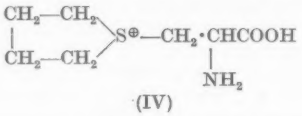
This work was a prelude to an investigation of the *in vivo* reactions of the more complex drug 1:4-dimethanesulphonyloxybutane (Myleran) (III),

$$CH_3SO_2O[CH_2]_4OSO_2CH_3$$

(III)

a cytotoxic alkylating agent of clinical use in the treatment of myeloid leukaemia. 1:4-Dimethanesulphonyloxy[2:3-$^{14}C_2$]butane (synthesized by Dr P. Brookes) was shown by radioautography of paper chromatograms to be excreted in the urine as one major metabolite.

A study of the *in vitro* reaction between cysteine and Myleran in an aqueous medium indicated that while some 1:4-dicysteinylbutane and *S*-hydroxybutylcysteine were formed, the major product of reaction was the *S*-β-alanyltetrahydrothiophenium ion (IV) which was characterized as its picrate.



(IV)

The urine of a rat, injected with the sulphonium salt (IV) prepared from the radioactive Myleran was shown to contain the major radioactive metabolite identical with that formed from Myleran. Metabolites of 1:4-dicysteinylbutane or *S*-hydroxybutylcysteine were not found in the urine of rats injected either with (IV) or with Myleran.

It is considered that an ion such as (IV) could be formed by *in vivo* reaction with the thiol group of cysteine or with cysteine bound in peptides or proteins. In rats injected with 1:4-dimethanesulphonyloxy[2:3-$^{14}C_2$]butane only approximately 4% of the injected radioactivity was exhaled as labelled carbon dioxide, whereas butane-1:4-diol which would be formed by hydrolysis of Myleran, is excreted almost entirely in the form of exhaled carbon dioxide.

The radioactivity in a number of selected organs at various times after injection of the radioactive drug was determined.

REFERENCES

Roberts, J. J. & Warwick, G. P. (1957). *Nature, Lond.*, **179**, 1181.

Roberts, J. J. & Warwick, G. P. (1958). *Biochem. Pharmacol.* **1**, 60.

The Oxidation–Reduction Potential of Cytochrome *b* in Heart-Muscle Preparation. By Johanna Colpa-Boonstra and F. A. Holton. (*M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London and Laboratory of Physiological Chemistry, University of Amsterdam, The Netherlands*)

The redox potential of cytochrome *b* is generally quoted as −40 mv at pH 7·4. This is the value found by Ball (1938), who employed visual spectroscopy to study the equilibrium between the pigment in heart-muscle particles and the succinate/fumarate system. We have re-studied this equilibrium in suspensions of similar particles prepared from horse and pig heart by the method of Keilin & Hartree (1949). Extinction changes due to pigment were measured spectrophotometrically (Holton, 1957). The results were calculated by rearranging the equation for the equilibrium constant

$$K = \frac{[\text{Fumarate}^{2-}]\,[b^{2+}]^2}{[\text{Succinate}^{2-}]\,[b^{3+}]^2}$$

to give

$$\frac{1}{[b^{2+}]} = \frac{1}{[b]} + \frac{1}{[b]\sqrt{K}}\sqrt{\frac{[\text{Fumarate}^{2-}]}{[\text{Succinate}^{2-}]}},$$

where $[b] = [b^{2+}] + [b^{3+}]$.

Values of K were calculated from a plot of $1/\Delta E_\lambda$ against $\sqrt{\{[\text{Fumarate}^{2-}]/[\text{Succinate}^{2-}]\}}$, where ΔE_λ refers to the observed extinction change associated with the reduction of cytochrome *b* from its oxidized state to its anaerobic equilibrium with succinate/fumarate. The redox potential of cytochrome *b* was calculated from observed values of K and the potential of the succinate/fumarate system, +24 mv at pH 7, 25° (Borsook & Schott, 1931). Interference by cytochromes *a*, a_3, *c* and c_1 was avoided by working at isosbestic points of these components taken together (434 and 558 mμ, determined by the method of Holton, 1955). Interference by myoglobin (Colpa-Boonstra & Minnaert, 1959) was eliminated by treating the particles with nitrite (Chance, 1952*a*).

In six experiments (three at each of the two wavelengths employed) we obtained values of E'_0, pH 7, 25° ranging from +60 to +90 mv, with a mean value of +77 mv. This result agrees with the comment by Hill (1954) that E'_0 is more positive than is generally accepted. Our equilibrium data agree closely with recently published α-band measurements in cyanide-treated material (Chance, 1958), but in disagreement with this author we conclude that under our experimental conditions a true equilibrium is established between cytochrome *b* and the succinate/fumarate system. It seems likely that the discrepancy between the present measurements and Ball's (1938) result is due to his use of dithionite. In agreement with Chance (1952*b*, 1958) we find that dithionite reduces an amount of cytochrome *b* or of a similar pigment which is not available for reduction by succinate.

REFERENCES

Ball, E. C. (1938). *Biochem. Z.* **295**, 262.
Borsook, H. & Schott, H. F. (1931). *J. biol. Chem.* **92**, 535.
Chance, B. (1952*a*). *J. biol. Chem.* **197**, 557.
Chance, B. (1952*b*). *Nature, Lond.*, **169**, 215.
Chance, B. (1958). *J. biol. Chem.* **233**, 1223.
Colpa-Boonstra, J. P. & Minnaert, K. (1959). *Biochim. biophys. Acta* (in the Press).
Hill, R. (1954). *Nature, Lond.*, **174**, 501.
Holton, F. A. (1955). *Biochem. J.* **61**, 46.
Holton, F. A. (1957). *Biochem. J.* **66**, 37P.
Keilin, D. & Hartree, E. F. (1949). *Biochem. J.* **44**, 205.

Recovery of Intravenously Injected Protein Antigens from Rat Spleens. By J. D. HAWKINS* and F. HAUROWITZ. (*Department of Chemistry, Indiana University, Bloomington, Indiana, U.S.A.*)

The distribution of injected antigens in the body has been widely studied (Haurowitz, 1953; Coons, 1954). There is little information about the chemical nature of the antigen remaining in the organ, though Garvey & Campbell (1957) have found fragments of the antigen in the ribonucleoprotein (RNP) fraction.

We have injected rats intravenously with either bovine γ-globulin (BGG) or bovine serum albumin (BSA), labelled with diazotized [^{35}S]sulphanilic acid. These rats were killed in groups of two or three 4–18 days later, and the spleens rapidly removed, homogenized and extracted twice with 0·10M-NaCl–0·05M-sodium citrate and thrice with distilled water, pH 7·0 (Chargaff, 1955). The 'free' antigen in a portion of each extract was precipitated with the appropriate rabbit antiserum in the presence of unlabelled carrier antigen and the ^{35}S in these precipitates determined. Non-specific absorption on these precipitates was shown to be negligible by simultaneously adding the unrelated ovalbumin-anti-ovalbumin system to other portions of the extracts. Following injection of either antigen about half the ^{35}S in the spleen was extractable by 0·1M-NaCl–0·05M-sodium citrate: of this about 70% was specifically precipitable by antibody during the 7 days after injection with BGG; thereafter less than 15% was precipitable.

In all rats injected with BSA only 3–10% of the extractable antigen was precipitable. Portions of some of the extracts were acidified to pH 4·5 using the procedure of Kay & Dounce (1953), which precipitates RNP, and the precipitates all contained ^{35}S. The amount of ^{35}S increased from 20% or less of the total extractable activity 4 days after injection of BGG to about 50% by 10 days. After injection of BSA activity appeared more rapidly in the RNP.

Only 8–15% of the ^{35}S in the spleen was found in the aqueous extracts (deoxyribonucleoprotein fraction). Purification of this fraction by precipitation with 0·15M-NaCl and re-solution in H_2O decreased this further.

Apparently part of the injected antigens or of their ^{35}S-containing fragments are bound to some constituent(s) of the spleen, rendering them unavailable for precipitation with antibody. There is some evidence that this unreactive antigen or antigen fragment is associated with RNP as suggested by Garvey & Campbell (1957).

* Present address: Department of Biochemistry, Medical College of St Bartholomews Hospital, London, E.C. 1.

REFERENCES

Chargaff, E. (1955). In *The Nucleic Acids* (ed. E. Chargaff & J. N. Davidson, New York), **1**, 307.
Coons, A. H. (1954). *Annu. Rev. Microbiol.* **8**, 333.
Garvey, J. S. & Campbell, D. H. (1957). *J. exp. Med.* **105**, 361.
Haurowitz, F. (1953). *Annu. Rev. Microbiol.* **7**, 389.
Kay, E. R. M. & Dounce, A. L. (1953). *J. Amer. chem. Soc.* **75**, 4041.

The Relationship between Cobalamin and Methionine Synthesis in *Escherichia coli* PA15. By J. R. GUEST (introduced by D. D. WOODS). (*Microbiology Unit, Department of Biochemistry, Oxford*)

Ultra-sonic extracts of *Escherichia coli* PA15, a serine or glycine mutant, have an absolute requirement for cobalamin in the synthesis of methionine from homocysteine and serine or (formaldehyde) when tetrahydropteroylglutamate (PtH_4G) is the cofactor for transfer of the one-carbon unit (Woods, 1958). The concentration of cyanocobalamin for half-maximal activity in dialysed extracts is 2×10^{-8}M. Several nucleotide-substituted cobalamin analogues can replace cyanocobalamin for promotion of methionine synthesis but factor B will not. The anilide of the monocarboxylic acid of cyanocobalamin (Smith, Parker & Gant, 1956) competitively inhibits the action of cyanocobalamin.

No cyanocobalamin is required for the synthesis of methionine under similar conditions if the

source of enzymes is an aqueous extract of an acetone-powder of organisms harvested from a medium supplemented with cyanocobalamin. However, similar extracts from organisms grown without cyanocobalamin are inactive even when the vitamin is added to the reaction mixture (Kisliuk & Woods, 1958). Such inactive preparations are caused to synthesize methionine (PtH_4G as cofactor) by the addition of a heat-labile, non-dialysable factor which can be obtained either, (*a*) from acetone powders of organisms harvested after growth with cyanocobalamin, or (*b*) by incubating ultra-sonic extracts with cyanocobalamin (Woods, 1958; Kisliuk & Woods, 1959).

Best production of the factor by dialysed ultrasonic preparations is found to require cyanocobalamin (10 μmg./mg. protein), ATP, DPN, Mg^{2+}, potassium phosphate (pH 7·8) and glucose; PtH_4G is not required. The anilide of cyanocobalamin inhibits the formation of this factor at similar concentrations to those at which it prevents synthesis of methionine. The action of the factor itself, however, is not inhibited by the anilide; this is the case with material formed by either method (*a*) or (*b*) above.

Addition of the factor restores methionine synthesis by ultra-sonic extracts inhibited by the anilide.

A sample of the anilide of cyanocobalamin was kindly given by Dr E. Lester Smith, F.R.S.

REFERENCES

Kisliuk, R. L. & Woods, D. D. (1958). *J. gen. Microbiol.* **18**, xv.

Kisliuk, R. L. & Woods, D. D. (1959). *Fed. Proc.* **18**, 261.

Smith, E. L., Parker, L. F. J. & Gant, D. (1956). *Biochem. J.* **62**, 14P.

Woods, D. D. (1958). Contribution to the discussion of paper by H. R. V. Arnstein, *Symposium (XI) on Vitamin Metabolism, IVth Int. Congr. Biochem., Vienna* (in the Press).

Incorporation of Amino Acids into the Proteins of the Isolated Microsome Fraction from Duck Livers. By P. N. Campbell and Barbara A. Kernot. (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W.* 1)

In 1954 Zamecnik & Keller showed that when the microsome fraction from rat liver is incubated with [^{14}C]amino acid, cell sap (105 000 *g* supernatant) and an adenosine triphosphate generating system, amino acids are incorporated into the microsomal protein. In order to study the specificity of the possible synthesis of serum albumin in such isolated preparations of liver microsomes it was desirable to extend our previous studies (Campbell, Greengard & Kernot, 1958) to non-mammalian livers.

In view of the work of Peters (1957) our attention was first directed to chick liver. However, when the microsomal fraction from chick liver was incubated with [^{14}C]amino acids under the conditions previously described for rat-liver microsomes (Campbell & Greengard, 1959) the energy-dependent incorporation of amino acid was negligible. A similar failure was experienced with pigeons and turkeys. However, the livers of young ducks provided an active fraction.

The microsomal fraction was isolated from duck liver as previously described for rat liver (Campbell & Greengard, 1959) except that the microsome pellet was not washed. When this fraction was incubated with cell sap and [^{14}C]amino acid, incorporation into protein took place in the presence of phosphoenolpyruvate (PEP) provided that the enzyme pyruvic kinase was added to the medium. In the latter requirement and in the enhanced incorporation in the presence of PEP compared with 3-phosphoglycerate the duck-liver microsomes differed from those of the rat. Under optimal conditions ^{14}C-labelled glycine, leucine and valine respectively were incorporated into the protein of liver microsomes from ducks and rats to a comparable extent.

The microsome pellet from duck livers differs from that of rat in appearance and may be readily separated into two components consisting of a light fluffy layer and a heavy clear gel. The separated light layer, in the presence of cell sap, retains the ability to incorporate amino acids although this is less than that of the whole pellet. The ribonucleic acid of the microsome pellet is concentrated in the light layer. Electron micrographs, kindly taken by Dr M. A. Epstein of the Bland Sutton Institute of Pathology, indicate that the light layer contains a considerable amount of rough endoplasmic reticulum with attached electron-dense particles, as well as a few mitochondria and other membranous structures.

REFERENCES

Campbell, P. N. & Greengard, O. (1959). *Biochem. J.* **71**, 148.

Campbell, P. N., Greengard, O. & Kernot, B. A. (1958). *Biochem. J.* **68**, 18P.

Peters, T. (1957). *J. biol. Chem.* **229**, 659.

Zamecnik, P. C. & Keller, E. B. (1954). *J. biol. Chem.* **209**, 337.

The Isolation of Phosphatidic Acid from Ox Liver. By G. Hübscher and B. Clark (introduced by R. R. Dils). (*Department of Medical Biochemistry and Pharmacology, The Medical School, University of Birmingham*)

Conflicting evidence exists concerning the occurrence of phosphatidic acid in animal tissues. Dawson (1954) and Marinetti & Stotz (1956) were unable to detect phosphatidic acid in animal tissues. Marinetti, Erbland, Albrecht & Stotz (1957) have described the occurrence of a compound resembling phosphatidic acid but having a higher P:ester ratio than that required for phosphatidic acid. The experiments of Hokin & Hokin (1958), using paper chromatography and ^{32}P-labelled phospholipids, indicated, however, that phosphatidic acid might occur in animal tissues. From time studies of the hydrolysis of total phospholipids, Hawthorne & Hübscher (1959) concluded that the small amounts of glycerophosphate isolated had most likely been derived from phosphatidic acid.

The present investigation describes the isolation of phosphatidic acid from freshly obtained ox liver. The minced liver was partially dried with acetone, extracted with chloroform–methanol and the extract taken to a small volume. This was then treated with 10 vol. of absolute alcohol at pH 8. The precipitate was dissolved in chloroform and phosphatidic acid was separated from the other phospholipids by chromatography on a silicic acid column using chloroform and mixtures of methanol and chloroform for elution. The phosphatidic acid was finally prepared as the sodium salt as described by Kates (1955). The yield of phosphatidic acid amounted to 0·5–1% of the total extracted phospholipids.

The preparation obtained was approximately 90% pure. The main contaminants were phosphatidyl serine and phosphatidyl ethanolamine, as shown by paper chromatography of the acid hydrolysate of the preparation. Inositol and choline could not be detected. The molar ratio of P:glycerol was 1:1·08, that of P:ester was 1:1·98 and that of P:N was 1:0·08. After mild alkaline hydrolysis of the phospholipid and chromatography of the resulting phosphoric acid esters on ion-exchange resin (Hawthorne & Hübscher, 1959), over 80% of the phosphoric acid ester applied to the resin was recovered as glycerophosphate.

The significance of these results with respect to the presence of phosphatidic acid *in vivo* will be discussed.

We are indebted to the M.R.C. (G.H.) and to the University of Birmingham (B.C.) for grants.

REFERENCES

Dawson, R. M. C. (1954). *Biochim. biophys. Acta*, **14**, 374.
Hawthorne, J. N. & Hübscher, G. (1959). *Biochem. J.* **71**, 195.
Hokin, L. E. & Hokin, Mabel R. (1958). *J. biol. Chem.* **233**, 800.
Kates, M. (1955). *Canad. J. Biochem. Physiol.* **33**, 575.
Marinetti, G. V., Erbland, J., Albrecht, M. & Stotz, E. (1957). *Biochim. biophys. Acta*, **26**, 130.
Marinetti, G. V. & Stotz, E. (1956). *Biochim. biophys. Acta*, **21**, 168.

On the Physiological Role of Diaphorase. By V. Massey. (*Department of Biochemistry, University of Sheffield*)

It has been shown recently that pig-heart diaphorase is a powerful lipoic dehydrogenase (Massey, 1958): reduced lipoic acid + DPN ⇌ oxidized lipoic acid + reduced DPN. Thus while reduced DPN can be oxidized by diaphorase, current theories on the role of lipoic dehydrogenase in keto acid oxidation (Reed, 1957; Gunsalus, 1954) envisage reduced DPN being formed by this enzyme in oxidative metabolism. The original work of Corran, Green & Straub (1939) suggest that the oxidation of reduced DPN is mediated by a flavoprotein, and that this flavoprotein is diaphorase. That the flavoprotein associated with the oxidation of reduced DPN is not identical with diaphorase is suggested by the fact that diaphorase can be extracted from a heart-muscle preparation with $(NH_4)_2SO_4$ without affecting the oxidation of reduced DPN by the particles. It was therefore of interest to determine whether the lipoic dehydrogenase of keto acid oxidation is also identical with diaphorase. The series of enzymes which bring about the oxidation of α-oxoglutarate in heart muscle appear to exist in the form of a molecular complex (Sanadi, Littlefield & Bock, 1952). This complex was isolated by the method of Sanadi *et al.* (1952) and further purified by a calcium phosphate gel-cellulose column fractionation. The complex was fluorescent and yellow, with an absorption spectrum very similar to that of diaphorase. A partial resolution of the complex into a colourless fraction which was active only in the presence of added diaphorase has been reported (Massey, 1959). Further proof

that the flavoprotein of α-oxoglutarate dehydrogenase is identical with diaphorase has come from a complete resolution of the complex into two fractions, one colourless, the other yellow, by fractionation in the presence of 2·5 M-urea. Separately, neither fraction catalyses the following oxidation of α-oxoglutarate:

α-oxoglutarate + CoA + DPN
→ succinyl CoA + CO_2 + reduced DPN,

but on mixing the two fractions, activity is restored. The yellow fraction exhibits all the physical, spectral and enzymic properties of diaphorase. The α-oxoglutarate dehydrogenase complex contains approximately equimolar amounts of thiamine pyrophosphate, lipoic acid and flavin (lipoic dehydrogenase). Of these protein-bound cofactors the thiamine pyrophosphate and lipoic acid are located in the colourless fraction. It would appear therefore that the enzymes concerned in the oxidation of α-oxoglutarate (one of which is diaphorase or lipoic dehydrogenase), are linked in stoicheiometric amounts in a functional enzyme complex, geared for the efficient oxidation of α-oxoglutarate.

REFERENCES

Corran, H. S., Green, D. E. & Straub, F. B. (1939). *Biochem. J.* **33**, 793.
Gunsalus, I. C. (1954). In McElroy, W. D. & Glass, B., *The Mechanism of Enzyme Action*, p. 545. Baltimore: The Johns Hopkins Press.
Massey, V. (1958). *Biochim. biophys. Acta*, **30**, 205.
Massey, V. (1959). *Biochim. biophys. Acta* (in the Press).
Reed, L. J. (1957). *Advanc. Enzymol.* **18**, 319.
Sanadi, D. R., Littlefield, J. W. & Bock, R. M. (1952). *J. biol. Chem.* **197**, 851.

Flavinogenesis in *Candida flareri*; the Role of Urea and Purines. By B. G. Audley, T. W. Goodwin and D. McEvoy. (*Biochemistry Department, The University, Liverpool*)

When urea replaces $(NH_4)_2SO_4$ as the N-source in a standard culture medium for *Candida flareri* there is a fourfold stimulation of both growth and flavinogenesis (Goodwin & McEvoy, 1957, 1959); however, replacement of $(NH_4)_2SO_4$ by certain amino acids (e.g. L-asparagine) causes a similar growth stimulation but no stimulation of flavinogenesis. These results suggested that urea participated in riboflavin biosynthesis either by incorporation into a purine-like precursor, for flavinogenesis and purine synthesis are closely related in other flavinogenic fungi (e.g. *Eremothecium ashbyii*) (Goodwin, 1959), or as an intermediate in a pathway not involving purines. The 'purine' and 'non-purine' pathways could conceivably be proceeding simultaneously.

Nutritional experiments with *C. flareri* showed that guanine considerably stimulates flavinogenesis whilst adenine has no significant effect (Goodwin & McEvoy, 1957, 1959). Experiments with [^{14}C]urea and [^{14}C]purines were undertaken to clarify the situation.

[^{14}C]Urea was added to 18 hr. shake cultures of *C. flareri* growing on our standard medium, that is just as flavinogenesis was beginning, and 12 hr. later the fermentation was stopped; during this period respiratory CO_2 was collected. Riboflavin isolated from the medium was purified by crystallization after addition of unlabelled carrier. Guanine and adenine isolated from the nucleic acids of the cells (Markham & Smith, 1950) were purified by paper chromatography. The distribution of counts in a typical experiment was: added (counts/min.) $11{\cdot}3 \times 10^6$; remaining in medium $7{\cdot}8 \times 10^6$; in CO_2 $1{\cdot}62 \times 10^6$; in cells 14 500; in riboflavin 0; in adenine and guanine 0. Thus urea-C is not incorporated into either purine nucleotides or riboflavin.

Generally labelled [^{14}C]adenine and [^{14}C]guanine, prepared from the blue-green alga *Anabaena cylindrica* cultured in the presence of $^{14}CO_2$, were examined in the same way as [^{14}C]urea. With [^{14}C]guanine 68 % of the added label was incorporated into riboflavin and activity was also recorded in the nucleic acid guanine but not adenine; with [^{14}C]adenine 16 % incorporation was obtained and activity was observed in both the nucleic acid adenine and guanine, the ratio of the activities in the two bases being approximately 3:2. These results demonstrate that purines can be incorporated effectively into riboflavin in *C. flareri* and indicate that there is little justification for concluding that the basic mechanism of flavinogenesis in this yeast differs significantly from that in the other micro-organisms which have been investigated.

This investigation was supported by the Agricultural Research Council.

REFERENCES

Goodwin, T. W. (1959). *Prog. industr. Microbiol.* **1**, 139.
Goodwin, T. W. & McEvoy, D. (1957). *Biochem. J.* **67**, 17.
Goodwin, T. W. & McEvoy, D. (1959). *Biochem. J.* (in the Press).
Markham, R. & Smith, J. D. (1950). *Biochem. J.* **46**, 513.

The Actions of Some Enzymes on Purine and its Derivatives. By G. R. BARKER, J. B. LLOYD, M. D. MONTAGUE and N. F. WOOD. (*Department of Chemistry, The University, Manchester*)

9-β-D-Ribofuranosylpurine administered to rats is partly converted into nucleic acid adenine and guanine, but no unchanged purine is found in the nucleic acids; purine riboside mono-, di- and triphosphate have been recognized in the acid-soluble fraction from viscera of treated animals (Gordon & Brown, 1956).

In order to interpret the metabolic fate of purine riboside in terms of the reactions it undergoes, the behaviour of purine derivatives towards appropriate enzymes has been studied. 9-β-D-Ribofuranosylpurine 5′-phosphate was converted by muscle adenylate kinase in the presence of ATP into the purine riboside diphosphate. Thus a mechanism exists for the formation of the polyphosphates observed by Gordon & Brown.

It is highly probable that RNA is formed in mammalian tissues by polymerization of nucleoside polyphosphates and a polynucleotide phosphorylase from human sperm has been partially purified (Hakim, 1959). The purine riboside diphosphate formed by the action of adenylate kinase was isolated and incubated with the polynucleotide phosphorylase of *Azotobacter vinelandii* (Grunberg-Monago, Ortiz & Ochoa, 1956). This enzyme failed to bring about phosphorolysis of the purine riboside diphosphate. If enzymes similar to the *Azotobacter* enzyme are present in rat tissues, the above result could account for the absence of unchanged purine in the nucleic acids of rats receiving purine riboside, and may in part explain the nature of the biological properties of purine derivatives.

In contrast to the riboside, purine does not give rise to purine riboside phosphates in the acid-soluble fraction (Gordon, Intrieri & Brown, 1957). Purine riboside has been subjected to nucleoside phosphorylase of calf spleen and the compound has been found not to be a substrate for this enzyme. It is believed that the inability of the nucleoside to undergo phosphorolysis and consequently to be formed from purine and ribose 1-phosphate may be partly responsible for the different metabolic fate of purine compared with that of the riboside and also for the high toxicity of the latter compound.

We wish to thank Dr G. B. Brown for the gift of purine riboside monophosphate.

REFERENCES

Gordon, M. P. & Brown, G. B. (1956). *J. biol. Chem.* **220**, 927.
Gordon, M. P., Intrieri, O. M. & Brown, G. B. (1957). *J. biol. Chem.* **229**, 641.
Grunberg-Monago, M., Ortiz, J. P. & Ochoa, S. (1956). *Biochim. biophys. Acta*, **20**, 269.
Hakim, A. A. (1959). *Nature, Lond.*, **183**, 334.

The Incorporation of ^{14}C into the Sorbitol of Plum Leaves. By J. D. ANDERSON, P. ANDREWS, and L. HOUGH. (*Department of Organic Chemistry, The University, Bristol* 8)

Sorbitol (D-glucitol) occurs widely in plants of the Rosaceae family, especially of the genera *Sorbus*, *Craetagus* and *Prunus* (Barker, 1955). About 5% of the dry weight of Victoria plum (*Prunus domestica*) fruit consists of sorbitol (Rees, 1958), and we have isolated similar amounts from the leaves. It is metabolized in pear and plum fruits on storage, possibly by oxidation to hexuloses (Donen, 1939; Kidd, West, Griffiths & Potter, 1938) and can be utilized for polysaccharide synthesis by Rosaceae and Gramineae leaves (Barker, 1955; Altermatt & Neish, 1956).

The incorporation of $^{14}CO_2$ into the sorbitol of mature plum leaves was investigated in an attempt to reveal details of its biosynthesis and metabolism, which are at present obscure. After being kept in the dark for 24 hr., the leaves were allowed to photosynthesize in an atmosphere containing, initially, about 5% $^{14}CO_2$ with carrier (Andrews & Hough, 1958). Since the $^{14}CO_2$ was completely incorporated in 8 hr., the leaves were then placed in the open. At various times samples were taken, and the leaf blades immediately extracted with hot methanol. After successive treatments of the soluble material with acid, sodium carbonate, and mixed-bed Amberlite IR-120(H) and IRA-400(OH) ion-exchange resins, in order to remove reducing sugars, sucrose, etc., the sorbitol was isolated by paper chromatography and converted into the crystalline hexa-*O*-acetate. No systematic variation in the sorbitol content of the leaves during photosynthesis was apparent, and quantitative estimations, by a modification of the method of Adcock (1957), of the hexitol in leaves removed from a tree at intervals over 24 hr. revealed that its concentration remained constant at 109 ± 4 mg./g. of methanol-insoluble leaf residue or 45 ± 2 mg./g. dry weight of leaf.

A high proportion of the ^{14}C supplied in $^{14}CO_2$ was incorporated into sorbitol, a maximum of

about 33% being reached after 15 hr. This value subsequently decreased to 15% after 48 hr., as a result of further metabolism. In the same experiments net synthesis of starch was occurring and the proportion of the supplied ^{14}C found in this polysaccharide was about 17 and 12%, after 24 and 48 hr., respectively. Clearly, the turnover of sorbitol in the leaf is considerable and proceeds at a rate quite comparable with that of starch, yet at the same time its concentration in the leaf does not vary.

Short-term experiments with leaf disks have further demonstrated the rapid synthesis of sorbitol following photosynthesis as well as the conversion of carbohydrates, labelled with ^{14}C in photosynthesis, into sorbitol, in the dark.

This work forms part of the programme of the Food Investigation Organisation of the Department of Scientific and Industrial Research.

REFERENCES

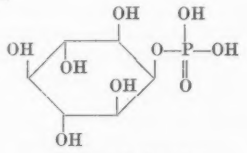
Adcock, L. H. (1957). *Analyst*, **82**, 427.
Altermatt, H. A. & Neish, A. C. (1956). *Canad. J. Biochem. Physiol.* **34**, 405.
Andrews, P. & Hough, L. (1958). *J. chem. Soc.* p. 4483.
Barker, S. A. (1955). *Modern Methods of Plant Analysis*, p. 55. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer-Verlag.
Donen, I. (1939). *Biochem. J.* **33**, 1611.
Kidd, F., West, C., Griffiths, D. G. & Potter, N. A. (1938). *Rep. Fd Invest. Bd, Lond.*, p. 132.
Rees, D. I. (1958). *J. Sci. Fd Agric.* **9**, 410.

_myo_Inositol 2-Phosphate, a Constituent of Liver Monophosphoinositide. By J. N. HAWTHORNE and G. HÜBSCHER. (*Department of Medical Biochemistry and Pharmacology, Medical School, Birmingham* 15)

Isolation of inositol monophosphate from inositides requires a hydrolysis which has recently been shown to cause phosphate migration (Posternak, 1958). For this reason it has been difficult to determine the position of the phosphate group on the inositol ring, though positions 2 or 5 have been suggested (Hawthorne, 1955).

Pure glycerylphosphorylinositol (GPI) has now been isolated from a hydrolysate of liver monophosphoinositide (Hawthorne & Hübscher, 1959). Hydrolysis of the barium salt of GPI in 0·5N-sodium hydroxide at 100° for 40 min. gave a mixture of inositol monophosphoric acid (IMP) and glycerophosphoric acid (GP). These esters were separated on columns of Nalcite SAR as described previously (Hübscher & Hawthorne, 1957). No inorganic phosphate was detected.

39 ± 2% of the phosphate of the hydrolysed GPI appeared as IMP and 61 ± 2% as GP. Paper chromatography in the two solvents used earlier (Hawthorne & Hübscher, 1959), followed by elution of the phosphoric acid esters and P determination, gave the same ratio. Comparison with the results of Brown, Hall & Higson (1958), who hydrolysed the *cis-* and *trans-cyclo*hexanediol analogues of GPI, shows that the above ratio is more likely to be obtained from GPI in which the inositol 2-phosphoric acid structure occurs, than from the 5-compound.



*myo*inositol 2-phosphate

That the proportion of IMP is not higher than 39% may be explained by the now usual postulation of a cyclic intermediate. Cyclization involving the 1- and 2-hydroxyls of the inositol would be hindered by the 3-hydroxyl, which is also in the *cis* configuration. The same effect is shown in the unusually difficult preparation of *iso*propylidene *myo*inositol.

REFERENCES

Brown, D. M., Hall, G. E. & Higson, H. M. (1958). *J. chem. Soc.* p. 1360.
Hawthorne, J. N. (1955). *Biochim. biophys. Acta*, **18**, 389.
Hawthorne, J. N. & Hübscher, G. (1959). *Biochem. J.* **71**, 195.
Hübscher, G. & Hawthorne, J. N. (1957). *Biochem. J.* **67**, 523.
Posternak, T. (1958). *Helv. chim. Acta*, **41**, 1891.

The Inhibition of Soluble Aconitase by Fluorocitric Acid. By R. A. PETERS. (*Biochemistry Department, A.R.C. Institute of Animal Physiology, Babraham, Cambridge*)

We have stated that soluble aconitase is much less easily inhibited than the aconitase in kidney mitochondria by fluorocitric acid synthesized by enzymic action (Morrison & Peters, 1954). This has been confirmed recently for Morrison's highly purified aconitase. The tests are done by observing the formation of *cis*-aconitate from *iso*citrate. However, a less purified soluble preparation shows quite different sensitivity, especially when the conditions of the tests are specially modified. To obtain a much larger inhibition, the test should be done upon aconitase at the stage of the '1st alcohol precipitation' in 'tris' buffer at pH 7·7 with a 10 min. incubation in buffer solution alone before addition of *iso*citrate. In this way, the effect of 0·01 μg. of the fluorocitric acid can be detected in the usual volume of 2·75 ml. The factors responsible for the difference are not accurately known; but the main difference lies in the fact that the enzyme can be used at this stage without activation with ferrous iron and cysteine. The fact that an isolated aconitase preparation can show this high sensitivity makes unnecessary now any explanation based upon differential permeability or upon a different orientation of the enzyme in the mitochondria.

I am indebted to Miss P. Ivall for skilful technica assistance.

REFERENCE

Morrison, J. F. & Peters, R. A. (1954). *Biochem. J.* **56**, xxxvi.

The Effect of Vitamin B_{12} on the Reduction of Glutathione Disulphide in Human Erythrocytes. By P. C. JOCELYN. (*Department of Clinical Chemistry, University of Edinburgh*)

The work of Dubnoff (1950) suggests that the low levels of glutathione (GSH) observed in vitamin B_{12}-deficient rats or patients with pernicious anaemia (Ling & Chow, 1953; Register, 1954) are due to a failure in the mechanism for maintaining GSH in its reduced form. Raised levels of glutathione disulphide (GSSG) and a deficiency in one of the components of the GSSG reductase system would then be expected. This has been examined as follows: Human red cell haemolysates treated with glucose 6-phosphate (G6-P) and a catalytic amount of triphosphopyridine nucleotide (TPN) convert GSSG to GSH (Collier & McCrae, 1955), the difference in concentration of the latter (by the method of Grunerts & Phillips, 1951) before and after the reaction measuring the amount of disulphide reduced. This method shows that there is a quantitative reduction of added GSSG (up to 0·5 μmole/3 ml. of 1:10 haemolysate) following incubation at 37° for 30 min. Consequently, the treatment applied to haemolysates without the addition of GSSG allows the estimation of any GSSG occurring in erythrocytes.

Similarly, the amount of reduction of added GSSG when G6-P is omitted measures the concentration of substrates reducing TPN. The activity of the enzyme system involved is obtained from the initial rate of reduction of added GSSG. Moreover, the slow rate of reduction found when TPN is omitted estimates the erythrocyte TPN-TPNH concentration by comparison with the rate for haemolysates to which known amounts of TPN have been added.

Although in two cases of pernicious anaemia (untreated) these results were within the normal range, in a third more severe case they were as follows (figures in parentheses show the range for ten normal subjects): GSH, 230 μmoles/100 ml. cells (160–300); GSSG, 55 μM/100 ml. cells (nil–15); reducible substrate, 70 μmoles/100 ml. cells (27 ± 13); enzyme activity (with 0·5 μM GSSG and G6-P, 0·1 μM TPN, 1 ml. of 1:10 haemolysate at pH 7·4 in a total volume of 4·5 ml.), 0·27 μM GSH produced in 6 min. at 37° and unaffected by added vitamin B_{12} (0·29–0·50); TPN–TPNH, 7 μmoles/100 ml. cells ($1{\cdot}1 \pm 6$). Three days after administration of vitamin B_{12} (1000 μg.) the values were: GSH, 333 μM; GSSG, nil; reducible substrate, 15 μM; other findings not significantly changed.

These results indicate that vitamin B_{12} is involved in the reduction of GSSG in human erythrocytes but do not show how this effect is exerted.

REFERENCES

Collier, H. B. & McCrae, S. C. (1955). *Canad. J. Biochem. Physiol.* **33**, 404.
Dubnoff, J. W. (1950). *Arch. Biochem. Biophys.* **27**, 466.
Grunerts, R. R. & Phillips, P. H. (1951). *Arch. Biochem. Biophys.* **30**, 217.
Ling, C. T. & Chow, B. F. (1953). *J. biol. Chem.* **202**, 445.
Register, U. D. (1954). *J. biol. Chem.* **206**, 705.

Metabolism of γ-Glutamyl Peptides in Subcellular Fractions of Rabbit Liver. By E. E. Cliffe and S. G. Waley. (*Nuffield Laboratory of Ophthalmology, University of Oxford*)

The subcellular fractionation of the enzymes catalysing the synthesis, hydrolysis and transpeptidation of glutathione (GSH) and ophthalmic acid (an analogue of GSH in which the thiol group is replaced by a methyl group) has been carried out.

The synthesis of ophthalmic acid in rabbit liver is catalysed by enzymes present in the soluble supernatant fraction; the other subcellular fractions showed little activity. The presence of cysteine depressed the rate of synthesis of ophthalmic acid, so it is probable that, as is the case with calf lens (Cliffe & Waley, 1958), one or both of the enzymes of glutathione synthesis also takes part in the synthesis of ophthalmic acid.

The enzyme catalysing the hydrolysis of the γ-glutamyl bond of GSH was localized in the nuclear fraction. Breakdown of ophthalmic acid also takes place in the nuclear fraction; moreover, the rate of breakdown of ophthalmic acid by the nuclear fraction is approximately halved if GSH is also present. This suggests that it is the same enzyme which is responsible for the hydrolysis of the γ-glutamyl bond in both GSH and ophthalmic acid. The nuclear fraction also catalyses transpeptidations; thus GSH and α-amino-*n*-butyrylglycine (ABGly) give ophthalmic acid:

γ-Glu Cys Gly + ABGly → γ-Glu ABGly + Cys Gly

It is quite likely that the one enzyme catalyses the hydrolysis and transpeptidation of both tripeptides, GSH and ophthalmic acid; this enzyme does not attack the dipeptide, γ-glutamylglycine, at a comparable rate.

The dipeptide, however, is cleaved by γ-glutamyl lactamase (Connell & Hanes, 1956). This enzyme is present exclusively in the supernatant fraction, and so its distribution parallels that of the enzymes which catalyse the synthesis of the tripeptides, and also that of the glycolytic enzymes concerned with ATP synthesis. As the synthesis of the tripeptides proceeds via γ-glutamyl dipeptides, the rate of synthesis *in vivo* will be affected by the presence of γ-glutamyl lactamase.

It is noteworthy that 96% of the protein in calf lens is in the supernatant fraction, and the enzymes that we detected previously in calf lens are now found to be those that we find in the supernatant fraction of the rabbit liver.

REFERENCES

Cliffe, E. E. & Waley, S. G. (1958). *Biochem. J.* 69, 649.

Connell, G. E. & Hanes, C. S. (1956). *Nature, Lond.*, 177, 377.

Demonstration of Auto-Antibodies Reacting with Human-Liver Lactic Dehydrogenase. By Pamela Burton, Lynda M. H. Kerr and S. C. Frazer. (*Department of Clinical Chemistry, University of Edinburgh*)

In acute hepatocellular lesions, enzymes derived from liver cells can be demonstrated in blood plasma (Molander, Wroblewski & LaDue, 1955). On the hypothesis that virus multiplication in the liver cell may render such proteins antigenic and capable of initiating auto-immunization phenomena, liver lactic dehydrogenase (LDH) has been selected as a model protein, and antibodies to human liver LDH have been sought in sera from patients suffering from acute and chronic hepatic disease.

Crystalline LDH has been prepared from human liver by the method described for rat liver LDH by Gibson and co-workers (Gibson, Davisson, Bachhawat, Ray & Vestling, 1953). Sera from patients with a variety of hepatocellular lesions produced no demonstrable inhibition of liver LDH activity *in vitro*. A modification of the haemagglutination technique (Boyden, 1951), using tannic acid-treated human erythrocytes (Group 'O', Rh-negative) has shown the presence of auto-antibody reacting with human liver LDH, in sera from a number of patients with hepatic cirrhosis. Sera from normal subjects, and from patients in the acute stage of virus hepatitis or of obstructive jaundice, have given negative results.

Attempts to initiate the production of similar antibodies in normal rats by multiple intravenous and intramuscular injections of crystalline rat liver LDH, with and without adjuvants (Freund & McDermott, 1942), have not so far produced demonstrable antibodies.

The results suggest, first, that the sites on the LDH molecule involved in antigen-antibody interaction do not coincide with those involved in its enzymic activity; and secondly, that the development of cirrhosis may be associated with the appearance of auto-immunization.

REFERENCES

Boyden, S. V. (1951). *J. exp. Med.* **93**, 107.
Freund, J. & McDermott, K. (1942). *Proc. Soc. exp. Biol., N.Y.*, **49**, 548.
Gibson, D. M., Davisson, E. O., Bachhawat, B. K., Ray, B. R. & Vestling, C. S. (1953). *J. biol. Chem.* **203**, 397.
Molander, D. W., Wroblewski, F. & LaDue, J. S. (1955). *J. Lab. clin. Med.* **46**, 831.

DEMONSTRATION

Micro-Tests for Sugars. By E. G. C. Clarke. (*Department of Physiology, Royal Veterinary College, London, N.W.* 1)

Microgram quantities of sugars may be identified by the conventional tests, using a technique similar to that described for the preparation of osazones (Clarke, 1957). Samples (5 μl.) of the solution are taken up in a melting-point tube, the indicated proportion of reagent added, and the mixture heated on the water bath for the time given below. With Benedict's reagent (1 vol.–1 min.), Bial's reagent (2 vol.–2 min.) and Selivanoff's reagent (3 vol.–5 min.) the usual colour changes are observed. In the case of Barfoed's reagent (1 vol.–3 min.) no change is visible to the naked eye, but star-shaped crystals of cuprous oxide may be seen under the microscope.

For the mucic acid test, a 2% solution of galactose or lactose is heated on the water bath in a sealed capilliary tube for 10 min. with an equal volume of concentrated nitric acid. The tube is then broken, and the contents transferred to a cavity slide and allowed to cool with occasional scratching. The crystals are best seen under polarized light.

REFERENCE

Clarke, E. G. C. (1957). *J. Physiol.* **135**, 28*P*.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **Annual General Meeting** *of the Society was held in the Department of Biochemistry, University College London, on Friday,* 3 *April* 1959. *The following papers were read:*

COMMUNICATIONS

Ultraviolet Fluorescence of Proteins. By F. W. J. Teale and G. Weber. (*Department of Biochemistry, The University of Sheffield*)

Tyrosine, tryptophan and phenylalanine have characteristic fluorescence in water solution (Teale & Weber, 1957). In the protein molecule the environment of the aromatic residues is modified in several ways: the surrounding medium is less polarizable than water, rotational freedom is restricted and adjacent groups of the polypeptide framework may take part in specific quenching processes. In addition, the average distance among the aromatic residues is about 10 Å, a distance at which energy transfer is known to take place in other systems (Förster, 1951). These possibilities have been examined in detail in solutions of twenty-six globular proteins. In all cases phenylalanine fluorescence was absent and that of tyrosine was observed only in the absence of tryptophan. In ribonuclease, insulin and zein tyrosine fluorescence with quantum yield of 0·03 on the average was present, while two trypsin inhibitors containing only tyrosine were non-fluorescent. Tryptophan fluorescence, and this fluorescence alone, was observed whenever this residue was present, with wide differences in absolute quantum yield and wavelength of maximum fluorescence emission. A shift of this maximum to shorter wavelength is accompanied by a comparable shift of the absorption maximum to longer wavelengths. Similar effects are shown by tryptophan in media of different polarizability. Viscous media (glycols) produced either no change (albumins) or large increase (trypsin, chymotrypsin) of the quantum yield. In contrast 8 M-urea lowered the quantum yield of the albumins but increased that of trypsin and chymotrypsin. The idea that the energy transfer of tyrosine to tryptophan accounts for the absence of tyrosine fluorescence was discarded by a study of the overall quantum yield as a function of the exciting wavelength. Excitation with light of 295–310 mμ, which is almost specific for the excitation of tryptophan, resulted in an increase in quantum yield indicative of the loss of the quanta absorbed by tyrosine when shorter wavelengths were used for excitation. It appears likely that tyrosine fluorescence is quenched by processes arising from secondary bonding involving the phenolic group. In five haem proteins tryptophan fluorescence appeared only upon removal of the prosthetic group. Evidently complete energy transfer to the haem takes place, a process suggested previously by the action spectrum of the photochemical dissociation of carboxymyoglobin (Bücher & Kaspers, 1946).

REFERENCES

Bücher, T. & Kaspers, J. (1946). *Naturwissenschaften*, **33**, 93.

Förster, Th. (1951). *Fluoreszenz Organischer Verbindungen*. Göttingen: Vandenhoeck and Ruprecht.

Teale, F. W. J. & Weber, G. (1957). *Biochem. J.* **65**, 476.

Polarization of the Ultraviolet Fluorescence and Electronic Energy Transfer in Proteins. By G. Weber and F. W. J. Teale. (*Department of Biochemistry, The University of Sheffield*)

The fluorescence of solutions of phenol, cresol and indole in propylene glycol at −70° in layers of 20–30 μ thickness showed concentration depolarization (Förster, 1951; Weber, 1954) over the range 0·01–0·1 M. The characteristic distances R for which the chances of transfer and emission are equal is for both phenol and indole 17 Å. Transfer in indole is only observed when the wavelength of excitation is shorter than 300 mμ. Even 0·4 M-indole solutions show strong polarization when excited with 310 mμ light. In mixtures of phenol and indole studied under the same conditions polarization and fluorescence spectral measurements show transfer from phenol to indole with $R = 20$ Å. Proteins that do not contain tryptophan (zein, insulin, ribonuclease), dissolved in 50 % propylene glycol–water mixtures at −70° and excited with light of 275–290 mμ wavelength show polarizations of

0·10–0·14, while cresol or phenol in 10^{-4} M solution show in the same conditions a polarization of 0·21. The depolarization observed in the proteins is fully consistent with the value of $R = 17$ Å deduced from the concentration depolarization of phenol or cresol fluorescence. The polarization of the fluorescence of proteins containing tryptophan (serum albumins, ovalbumin, chymotrypsin, γ-globulin, fumarase) do not show signs of depolarization by transfer of energy from tyrosine to tryptophan. Such transfer cannot have a quantum yield higher than 0·05. In lysozyme the values observed are consistent with transfer of 10–30 % of the energy among the tryptophan residues.

From the change in polarization with viscosity the lifetime of the excited state of tryptophan may be estimated as 4 mμ sec., that of tyrosine as 10 mμ sec. approximately. These values are too short to permit measurements of fluorescence depolarization to be of value in the determination of the rotational relaxation times of proteins in solution (Weber, 1952) but can give useful information on local conditions about the tryptophan or tyrosine residues.

REFERENCES

Förster, Th. (1951). *Fluoreszenz Organischer Verbindungen.* Chapter IX. Göttingen: Vandenhoeck and Ruprecht.
Weber, G. (1952). *Biochem. J.* **51**, 145, 155.
Weber, G. (1954). *Trans. Faraday Soc.* **50**, 552.

On the Intramolecular Location of Deoxyribonucleotides Incorporated into Deoxyribonucleic Acid by Extracts of Ascites Tumour Cells. By E. D. Gray and R. M. S. Smellie. (*Department of Biochemistry, The University, Glasgow, W. 2*)

The incorporation of [^{3}H]thymidine into deoxyribonucleic acid (DNA) by soluble preparations from mammalian cells has previously been described (Davidson, Smellie, Keir & McArdle, 1958; Smellie, Keir & Davidson, 1959). There are essentially three ways in which thymidine could be incorporated: (*a*) as a single terminal nucleotide on an existing polynucleotide chain; (*b*) as a polythymidylic acid extension to an existing polynucleotide; and (*c*) as a nucleotide randomly distributed in a completely new portion of a polynucleotide. Experiments were therefore designed to distinguish between these three possibilities.

The ^{3}H-DNA was digested with deoxyribonuclease II and spleen diesterase (Adler, Lehman, Bessman, Simms & Kornberg, 1958) to yield the four deoxyribonucleotides as 3′-phosphates. When they were separated, a high proportion of the radioactivity of the DNA was recovered as thymidine 3′-monophosphate. If the thymidine were incorporated as in (*a*), degradation in this fashion would liberate all radioactivity as thymidine. Hence a high proportion of the thymidine must be incorporated into non-terminal positions.

When labelled DNA was digested with 5′-diesterase, the rates of release of acid-soluble radioactivity and of nucleotides could be closely correlated. Since this enzyme degrades DNA in a stepwise fashion from the deoxynucleoside end of the chain, this result indicates (*c*) and precludes (*a*) and (*b*), otherwise all the radioactivity would have been liberated in the very early stages of the digestion.

In other experiments ^{32}P-labelled deoxycytidine and thymidine 5′-monophosphates (dCMP and TMP) were separately incorporated into DNA. The labelled DNA's were then degraded with deoxyribonuclease II and spleen diesterase to yield the deoxyribonucleoside 3′-monophosphates. With either dCMP or TMP as precursors, the radioactivity of the DNA was distributed approximately evenly among the four constituent nucleoside 3′-monophosphates. This indicates random distribution of the incorporated nucleotide in the polynucleotide and excludes the possibility of incorporation as in (*b*). Moreover, since one nucleotide appears to be incorporated for every 30 nucleotide residues, it was impossible to account for all the nucleotide incorporated in terms of single terminal nucleotides.

It is therefore concluded that the incorporation of thymidine represents synthesis of a new portion of a polynucleotide chain.

REFERENCES

Adler, J., Lehman, I. R., Bessman, M. J., Simms, E. S. & Kornberg, A. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 641.
Davidson, J. N., Smellie, R. M. S., Keir, H. M. & McArdle, A. H. (1958). *Nature, Lond.*, **178**, 514.
Smellie, R. M. S., Keir, H. M. & Davidson, J. N. (1959). *Biochim. biophys. Acta* (in the Press).

Net Synthesis of Deoxyribonucleic Acid by Extracts of Ascites Tumour Cells. By R. M. S. SMELLIE, H. M. KEIR, E. D. GRAY, D. BELL, J. RICHARDS and J. N. DAVIDSON. (*Department of Biochemistry, The University, Glasgow, W. 2*)

We have previously described conditions for the incorporation of [^{3}H]thymidine into deoxyribonucleic acid (DNA) by extracts of sonically disrupted ascites cells (Davidson, Smellie, Keir & McArdle, 1958). This paper describes the optimum conditions for thymidine incorporation and conditions in which net synthesis of DNA has been obtained in extracts of osmotically disrupted cells. The system described is similar to those of Bollum & Potter (1958) and Mantsavinos & Canellakis (1958) for regenerating rat liver and Bessman, Lehman, Simms & Kornberg (1958) for extracts of *Escherichia coli*.

For the incorporation of thymidine, ATP, Mg^{2+} ions and primer DNA are required; incorporation is promoted by low concentrations of the deoxyribonucleoside monophosphates and, to a greater extent, by the deoxyribonucleoside triphosphates of adenine, guanine and cytosine.

Net synthesis of DNA has been obtained in the presence of all four deoxyribonucleoside monophosphates, ATP and Mg^{2+} ions, an increase of 29% or 25 μg. of DNA per tube being observed. With all four deoxyribonucleoside triphosphates and Mg^{2+}, somewhat greater synthesis of 32% or 31 μg. DNA per tube is obtained.

REFERENCES

Bessman, M. J., Lehman, I. R., Simms, E. S. & Kornberg, A. (1958). *J. biol. Chem.* **233**, 171.
Bollum, F. J. & Potter, V. R. (1958). *J. biol. Chem.* **233**, 478.
Davidson, J. N., Smellie, R. M. S., Keir, H. M. & McArdle, A. H. (1958). *Nature, Lond.*, **178**, 514.
Mantsavinos, R. & Canellakis, E. S. (1958). *Biochim. biophys. Acta*, **27**, 661.

Enzymic Formation of Deoxyribonucleoside Triphosphates. By H. M. KEIR. (*Department of Biochemistry, The University, Glasgow, W. 2*)

Current theories on the mechanism of biosynthesis of deoxyribonucleic acid (DNA) suggest that the immediate precursors of DNA are deoxyribonucleoside triphosphates which are condensed enzymically to form deoxyribopolynucleotide with the concomitant release of inorganic pyrophosphate.

The system described by Smellie, Keir & Davidson (1959), which employs sonically or osmotically disrupted preparations of Ehrlich ascites tumour cells as the enzyme source, readily incorporates [^{3}H]thymidine (TDR) into DNA. It might therefore be expected that the TDR, together with other deoxyribonucleoside and deoxyribonucleotide precursors of DNA present in the system, are converted to the deoxyribonucleoside triphosphates prior to incorporation.

It has been found that ^{3}H-TDR is indeed phosphorylated during incubation with suitably fortified particle-free extracts of the tumour cells, radioactive thymidine 5′-mono-, di- and tri-phosphates (TMP, TDP and TTP) being formed.

The extracts, when incubated with TMP, deoxycytidine-5′-monophosphate (dCMP), deoxyadenosine-5′-monophosphate (dAMP) or deoxyguanosine-5′-monophosphate (dGMP) in the presence of adenosine triphosphate and Mg^{2+} ions at pH 8, are capable of producing the corresponding triphosphates (TTP, dCTP, dATP and dGTP). The yields of TTP and dCTP under the conditions used are low, but the conversion of dAMP and dGMP to dATP and dGTP respectively is of the order of 60–80%.

Ionophoresis and chromatography on paper have been used in the isolation and characterization of the deoxyribonucleoside triphosphates.

REFERENCE

Smellie, R. M. S., Keir, H. M. & Davidson, J. N. (1959). *Biochim. biophys. Acta* (in the Press).

Patterns of Oxidative Assimilation in *Sarcina lutea*. By BARBARA BINNIE, W. H. HOLMS and E. A. DAWES. (*Department of Biochemistry, The University, Glasgow, W. 2*)

The oxidation of glucose by washed suspensions of *Sarcina lutea* is accompanied by an extensive deposition of cellular carbohydrate (Dawes & Holms, 1958*a*) and a linear relationship has been established between the glucose utilized and the carbohydrate deposited. An investigation of oxidative assimilation has been carried out with variously labelled substrates.

Time-course studies have shown that approximately 55% of the radioactivity added as [U-^{14}C]-

glucose appears within the cells and that when the external glucose is exhausted this value falls slightly, presumably as a result of the oxidation of some labile intermediates. With [1-^{14}C]glucose the assimilated radioactivity is rather less, and this is referable to the fact that the pentose phosphate cycle accounts for the oxidation of about 30% of the substrate glucose, the remainder being metabolized via the glycolytic pathway (Dawes & Holms, 1958*b*, *c*). The oxidation of [3:4-$^{14}C_2$]-glucose results in the assimilation of approximately 54% of the added radioactivity.

The metabolism of glucose by *S. lutea* yields both pyruvate and acetate and the assimilation of these compounds was therefore also studied. Oxidation of pyruvate labelled in the 1-, 2- and 3-carbon atoms yielded respectively 7, 40 and 50% of the added radioactivity within the cells. These findings indicate that the carboxyl group is largely eliminated prior to assimilation, a finding previously reported with lactate for *Escherichia coli* (Fraser & Tolbert, 1951) and *Pseudomonas saccharophila* (Wiame & Doudoroff, 1951). This belief is supported by the assimilatory pattern obtained with [1-^{14}C]- and [2-^{14}C]-acetate and is also in accord with the operation of the tricarboxylic acid cycle.

An interesting feature of the results is that although the metabolism of [3:4-$^{14}C_2$]glucose via both glycolytic and pentose phosphate pathways yields [1-^{14}C]pyruvate, the percentage radioactivity assimilated from these substrates is 54 and 7 respectively. Furthermore, analyses of cells after assimilation of pyruvate and acetate revealed no significant deposition of carbohydrate. These observations demonstrate that the product(s) of pyruvate and acetate assimilation differ from those of glucose and that the carbon atoms of glucose are not assimilated via pyruvate.

The rates of utilization and assimilation of pyruvate and acetate are suppressed by the presence of glucose, an effect which persists until the glucose is almost exhausted.

REFERENCES

Dawes, E. A. & Holms, W. H. (1958*a*). *Biochim. biophys. Acta*, **30**, 278.
Dawes, E. A. & Holms, W. H. (1958*b*). *J. Bact.* **75**, 390.
Dawes, E. A. & Holms, W. H. (1958*c*). *Biochim. biophys. Acta*, **29**, 82.
Fraser, D. & Tolbert, B. (1951). *J. Bact.* **62**, 195.
Wiame, J. M. & Doudoroff, M. (1951). *J. Bact.* **62**, 187.

Interaction between Pyridoxal Phosphate and Amino Acids. By H. K. King and N. Lucas. (*Department of Biochemistry, The University, Liverpool* 3)

Most pyridoxal phosphate enzymes are believed to act through union between the –CHO of the coenzyme and the –NH_2 of an amino acid, forming a Schiff base (Blakley, 1955) which in some cases may chelate with a metal (Metzler, Ikawa & Snell, 1954; Matsuo, 1957). Classical Michaelis constants of such enzymes might be expected to be of the same order as the stability constants for the pyridoxal phosphate–amino acid compounds if expressed in similar units. Typical Michaelis constants (K_s) reported for enzymes of this type are: glutamic decarboxylase (bacterial), pH 4·5, 0·027M; (plant), pH 5·6, 0·004M; (brain), pH 6·3, 0·006M; transaminase (liver), pH 7, alanine 0·015M, glutamic acid, 0·014M; leucine/valine decarboxylase (bacterial), pH 6, 0·01M; tryptophanase (bacterial), pH 7·5, 0·0004M.

This communication reports the stability of some pyridoxal phosphate–amino acid compounds in terms analogous to the Michaelis constants, viz. stability (K) = concentration of amino acid required to convert half of the pyridoxal phosphate present to the Schiff base under specified conditions comparable with those obtaining in enzyme systems. Pyridoxal phosphate (10^{-4}M) in buffer was treated with varying concentrations of the amino acid and Schiff-base formation followed by changes in absorption spectrum in the 280 and 410 mμ regions.

Valine. Stability increases with rise of pH from K = 0·05M at pH 4·5 to K = 0·007M at pH 7. Addition of $CoCl_2$ (10^{-3}M; sufficient to give maximal chelate complex formation) increases stability of the Schiff base only slightly. Cobalt was selected since Matsuo (1957) reported its complexes to be the most stable from a wide range of metals examined.

Glutamic acid. Little change over the range pH 4 (K = 0·04M) to pH 7 (K = 0·02M). Co^{2+} had a marked effect at the higher pH values; K = 0·04M at pH 4; 0·003M at pH 6; 0·001M at pH 7.

Glycine. Little affinity at acid pH (K = 0·36M at pH 5·5); greater stability (K = 0·03M) at pH 7.

REFERENCES

Blakley, R. L. (1955). *Biochem. J.* **61**, 315.
Matsuo, Y. (1957). *J. Amer. chem. Soc.* **79**, 2011.
Metzler, D. E., Ikawa, M. & Snell, E. E. (1954). *J. Amer. chem. Soc.* **76**, 648.

Aryl Sulphamate Synthesis. By A. B. Roy. (*Department of Biochemistry, University of Edinburgh*)

It has recently been demonstrated that enzyme preparations from mammalian liver (Roy, 1958) and sheep intestinal mucosa (Kent & Pasternak, 1958) can synthesize aryl sulphamates from certain arylamines in the presence of SO_4^{2-} ions and ATP. The present communication describes results obtained in a study of this synthesis using quantitative methods. The enzyme preparations were obtained from liver by methods previously described (Roy, 1956).

The optimum conditions for the synthesis of 2-naphthyl sulphamate by rat or guinea-pig liver enzymes were as follows: optimum pH, 8·2–8·3; optimum ATP concentration, 4 mM; optimum SO_4^{2-} concentration, 10 mM. The optimum substrate concentration was greater than 2 mM 2-naphthylamine, the greatest practical concentration because of the low solubility of this amine at pH 8·2.

It was expected that the synthesis of 2-naphthyl sulphamate would involve sulphate transfer from adenosine - 3′ - phosphate - 5′ - sulphatophosphate (PAPS) catalysed by a specific sulphokinase, arylamine sulphokinase. PAPS was prepared enzymically and it was shown that sulphate is transferred therefrom to 2-naphthylamine by a sulphokinase present in liver. The sulphokinase reaction has an optimum pH of 8·2–8·3 and requires the presence of Mg^{2+} ions for optimal activity. In the absence of Mg^{2+} ions the activity is only some 40% of the optimal: this would seem to distinguish arylamine sulphokinase from phenol sulphokinase (Gregory & Lipmann, 1957). Arylamine sulphokinase is an SH enzyme as it is reversibly inhibited by *p*-chloromercuribenzoate. It has not so far been separated from other sulphokinases present in the preparation, but comparative studies show its separate identity.

It has been found that the synthesis of 2-naphthyl sulphamate by rat liver preparations, but not by guinea-pig liver enzymes, is strongly activated by certain steroids. *epi*Androsterone at a concentration of 10^{-5} M increases the amount of sulphamate formed by approximately 400%. Of a large number of steroids tested only those with an oxo group in the 17 position caused this activation, which occurred either with the complete enzyme system or with the sulphokinase-catalysed transfer from PAPS. It is suggested that the activation depends upon the formation of the 17-enol sulphate of the appropriate steroid, in which the sulphate group potential would be expected to be high, with subsequent transfer of this sulphate to 2-naphthylamine by a reaction which does not involve arylamine sulphokinase. The latter cannot be involved as it is rate limiting in the rat enzyme system.

REFERENCES

Gregory, J. D. & Lipmann, F. (1957). *J. biol. Chem.* **229**, 1081.
Kent, P. W. & Pasternak, C. A. (1958). *Abstr. 4th Int. Congr. Biochem., Vienna*, p. 47.
Roy, A. B. (1956). *Biochem. J.* **63**, 294.
Roy, A. B. (1958). *Biochim. biophys. Acta*, **30**, 193.

Photosynthetic Uptake of Labelled Carbon Dioxide by Sub-Cellular Components of Spinach Leaves. By V. Moses,* O. Holm-Hansen,† N. G. Pon, K. Nishida‡ and M. Calvin. (*Radiation Laboratory and Department of Chemistry, University of California, Berkeley 4, California, U.S.A.*)

Chloroplasts, mitochondria and sap were separated from spinach leaves, and were allowed to carry on photosynthesis for 30 min. in the presence of $NaH^{14}CO_3$, either alone or in various combinations. The ^{14}C fixed by chloroplasts alone was distributed mainly among the phosphate esters, while that fixed by the sap was located largely in amino and organic acids. The mitochondrial fraction fixed very little ^{14}C and was bright green in colour, being grossly contaminated with chloroplast fragments which could not be removed by differential centrifugation.

While chloroplasts fixed about five times as much ^{14}C as sap, the recombined mixture of chloroplasts and sap fixed three times more ^{14}C than the sum of the separated components. The distribution pattern of fixed ^{14}C in the recombined mixture was qualitatively similar to a combination of the distributions with the separated chloroplasts and sap. The addition of boiled sap to chloroplasts, or of mitochondria to the chloroplast-unboiled sap mixture, had little or no effect on the uptake and distribution of ^{14}C. In the dark, a mixture of all three components fixed about ten times less $^{14}CO_2$ than in the light, and ^{14}C was found predominantly in amino and organic acids.

Owing to the relative inactivity of the mitochondrial preparation, it was not possible to study the role of the sap as a carrier of carbon compounds between the chloroplasts and the mitochondria.

* Present address: Department of Chemical Pathology, King's College Hospital Medical School, London, S.E. 5.
† Present address: Department of Botany, University of Wisconsin, Madison 5, Wisconsin, U.S.A.
‡ Present address: Botanical Institute, Kanazawa University, Kanazawa, Japan.

The sap has been shown to contain glycolytic enzymes, but no carbon reduction cycle activity, and the chloroplasts no tricarboxylic acid cycle activity, so that some spatial separation of activities seems likely in the complete cell. The catalytic effect of the sap was probably enzymic, and the explanation for this phenomenon may be that the true substrate for carboxydismutase is neither carbon dioxide nor bicarbonate ion, but an activated form of carbon dioxide which is formed in the sap and then taken up by the chloroplasts.

This work was sponsored in part by the U.S. Atomic Energy Commission, and in part by the Department of Chemistry, University of California, Berkeley, California.

The Possible Conversion of Fat into Carbohydrate in the Obese Human. By G. L. S. PAWAN. (*Medical Unit, Middlesex Hospital, London, W.* 1)

Although tissues of higher animals are known to be capable of incorporating fatty-acid carbon into carbohydrate, net conversion of fat to carbohydrate still remains in doubt (Weinman, Strisower & Chaikoff, 1957).

From studies of R.Q. values in obese humans it was suggested (Lyon, Dunlop & Stewart, 1932) that fat conversion to carbohydrate may occur in these subjects. It had also been observed (Kekwick & Pawan, 1957) that obese patients showed rapid weight loss on a high fat diet (1000 kcal., 10 g. NaCl, 3 l. water per day, of which 90% of the calories were supplied in the form of fat) for periods up to 3 weeks, but seldom exhibited marked ketonaemia or acidosis. The carbohydrate and protein contents of this diet were only 12 and 16 g. respectively, yet blood sugar was maintained within normal limits, and during this time, body protein, as assessed from nitrogen balance, was not greatly affected. Three apparently normal non-obese subjects after a few days on this type of diet, developed severe ketosis, hypoglycaemia and negative nitrogen balance.

It would appear from these results that in the obese human on a high fat, low calorie, carbohydrate restricted diet, mechanisms of fat catabolism are considerably more efficient than in the non-obese human with respect to economy of body protein and carbohydrate stores, due to possible adaptation to a high fat metabolic mixture (Pawan, 1957) and recently reported in the rat on a high fat diet (Mayes, 1959), or alternatively, that gluconeogenesis from fat occurs under these conditions as a result of adaptation of metabolic pathways along some other channels, e.g. the glyoxylate cycle (Kornberg & Krebs, 1957), or lactate formation from acetoacetate with net gain of carbon (Ottaway & Sarkar, 1958).

REFERENCES

Kekwick, A. & Pawan, G. L. S. (1957). *Metabolism*, **6**, 447.
Kornberg, H. L. & Krebs, H. A. (1957). *Nature, Lond.*, **179**, 988.
Lyon, D. M., Dunlop, D. M. & Stewart, C. P. (1932). *Biochem. J.* **26**, 1107.
Mayes, P. A. (1959). *Biochem. J.* **71**, 459.
Ottaway, J. H. & Sarkar, A. K. (1958). *Nature, Lond.*, **181**, 1791.
Pawan, G. L. S. (1957). *Biochem. J.* **67**, 33P.
Weinman, E. O., Strisower, E. H. & Chaikoff, I. L. (1957). *Physiol. Rev.* **37**, 252.

Stimulation of Liver Acid Phosphatase Activity following the Parenteral Injection of Excessive Doses of Iron. By L. GOLBERG, L. E. MARTIN, C. M. BATES and A. BATCHELOR. (*Research Department, Benger Laboratories Ltd., Holmes Chapel, Cheshire*)

In seeking to define in biochemical terms the consequences which flow from excessive overloading of the body with iron, we have administered iron-dextran complex (IDC) and saccharated oxide of iron (SOI) parenterally to groups of 10 mice, rats or guinea pigs. The acid phosphatase activity (APA) of liver homogenates, using phenyl phosphate, is expressed as μg. P/100 mg. fresh liver/min.

In normal mice, the liver APA is $11{\cdot}5 \pm 2{\cdot}6$ for males and $17{\cdot}6 \pm 5{\cdot}5$ for females. In male mice, 6 hr. after a single intravenous dose of 450 mg. Fe/kg. as IDC, APA was $25{\cdot}2 \pm 4{\cdot}6$; 24 hr. after each of 1, 2, 3 and 9 daily injections of 220 mg. Fe/kg. APA was $24{\cdot}7 \pm 7{\cdot}0$, $33{\cdot}9 \pm 5{\cdot}1$, $39{\cdot}8 \pm 3{\cdot}9$ and $58{\cdot}0 \pm 13{\cdot}4$ respectively. In the last group the liver weight was $2{\cdot}7 \pm 0{\cdot}75$ g., making a total APA per liver of 1532 ± 139. A saline-treated control group had APA $12{\cdot}3 \pm 4{\cdot}2$, liver weight $1{\cdot}55 \pm 0{\cdot}17$ g. and total liver APA 191 ± 70. With low-molecular dextran APA did not rise (mean liver weight $1{\cdot}96 \pm 0{\cdot}43$ g.) Nine daily injections of SOI, 182 mg. Fe/kg., raised APA to $50{\cdot}3 \pm 11{\cdot}6$ and increased the mean liver weight by one-third. Rats gave similar results but guinea pigs showed no elevation of APA. Serum APA was unchanged in

any species. Of seven other liver enzymes, none showed changes of comparable magnitude to the APA. Analysis of the enlarged livers from treated mice showed no disproportionate increase in water, protein, carbohydrate, nucleic acid or mineral content (other than that attributable to iron). The RNA/DNA ratio indicates that the growth is due to hyperplasia, and not hypertrophy.

The elevation of liver APA in immature and adult mice and rats is thus a specific effect of heavy parenteral overdosage with iron. Ten weekly intramuscular injections of 5 mg. Fe/kg., a total still exceeding twice the average clinical dose of IDC, did not increase the liver weight or APA. When given daily intravenously, these doses increased liver weight by 58 % but did not significantly alter the APA.

Six months after heavy overloading with iron, the liver APA remains elevated, apparently representing a permanent change in the enzyme activity of the liver.

The authors are grateful to the Directors of Benger Laboratories Ltd. for permission to publish these results.

Succinyl Group-Translocation through the Plasma Membrane of *Micrococcus lysodeikticus*.

By P. MITCHELL and JENNIFER MOYLE. (*Zoology Department, University of Edinburgh*)

At a meeting of this Society nearly three years ago (Mitchell & Moyle, 1956), we presented the first unequivocal evidence for the presence of components of the cytochrome system and associated enzymes, not in intracellular particles but as an integral part of the plasma membrane of bacteria. The object of our studies was not simply to obtain data about the enzymic anatomy of bacteria, but, as discussed at this Society's Symposium in 1957, to obtain an orthodox biochemical explanation of the movement of metabolites across the membrane separating the outer medium from the cytoplasm (Mitchell, 1957, 1959).

Studies of the kinetics and specificity of succinate transport, and of the inhibitory action of 2:4-dinitrophenol, sodium arsenite, and phenylmercuric chloride on the transport process imply that, during exogenous succinate metabolism, it is the succinyl group and not the succinate molecule that passes into and through the membrane (Mitchell, 1959; Mitchell & Moyle, 1958). In this paper two distinct enzyme systems of *Micrococcus lysodeikticus* are described that produce hydroxamates from succinate and hydroxylamine. One of these systems (M) is found in the membrane, and the other (S) in the cytoplasm fraction. System M requires ATP and CoA, and resembles the succinate-activating system of *Escherichia coli*, for which the following reactions have been suggested (Smith, Frank & Gunsalus, 1957), see top of next column.

$$\text{ATP} + \text{CoA} \rightleftharpoons \text{ADP} + \text{CoA-P} \quad \text{(thiokinase)}$$

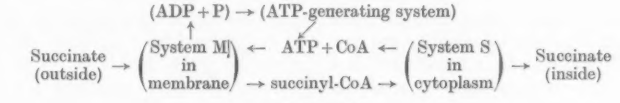
$$\text{CoA-P} + \text{succinate} \rightleftharpoons \text{succinyl-CoA} + \text{P} \quad \text{(phosphoryl-CoA transferase)}$$

The system S, unlike system M, requires neither CoA nor ATP for hydroxamate formation with succinate, and in this respect it resembles acetylcholine esterase which will form a hydroxamate with hydroxylamine and acetate alone (Hestrin, 1949). A preliminary comparison of the characteristics of the M and S systems with the characteristics of the succinate transport system of intact cells suggests that M and S are implicated as in Scheme 1.

This development of the group-translocation theory will be considered in the light of experiments designed to show whether the part of the M system which reacts with succinate is accessible from the medium on the outer side of the plasma membrane of intact organisms.

REFERENCES

Hestrin, S. (1949). *J. biol. Chem.* **180**, 249.
Mitchell, P. (1957). *Biochem. J.* **65**, 44P.
Mitchell, P. (1959). *Biochem. Soc. Symp.* **16**, 73.
Mitchell, P. & Moyle, J. (1956). *Biochem. J.* **64**, 19P.
Mitchell, P. & Moyle, J. (1958). *VII Int. Congr. Microbiol.* (*Abstracts*), p. 25.
Smith, R. A., Frank, I. F. & Gunsalus, I. C. (1957). *Fed. Proc.* **16**, 251.


(ADP + P) → (ATP-generating system)
Succinate (outside) →
System M in membrane
← ATP + CoA ←
System S in cytoplasm
→ Succinate (inside)
→ succinyl-CoA →

Scheme 1

The Effect of Isoniazid, Propamidine, Pentamidine and Carbutamide on the Growth and Transaminases of *Escherichia coli*. By R. MARIAN HICKS.* (*Department of Biochemistry, University College, Gower Street, London, W.C.* 1)

Rudman & Meister (1953) demonstrated three distinct transaminases in *Escherichia coli*, each capable of catalysing a number of transamination reactions. For the present experiments the aspartic acid/glutamic acid reaction was selected as representative of the reactions catalysed by Enzyme 1 and the valine/glutamic acid reaction as representative of Enzyme 2. The activities of these enzymes in cell-free extracts of a mutant of *E. coli*, strain 15, were investigated by estimation of the glutamic acid produced. Isoniazid is known to inhibit the aspartic acid/glutamic acid transaminase of pig heart muscle (Hicks & Cymmerman Craig, 1957), pentamidine to inhibit the phenylalanine/glutamic acid transaminase (Enzyme 2) of *E. coli* (Amos & Vollmayer, 1957) and carbutamide to inhibit rat liver transaminases (Bornstein, 1957).

Experiment showed that, of the diamidines, propamidine was without effect on either transaminase in *E. coli* and that 10^{-2}M-pentamidine produced 50% inhibition of the valine/glutamic acid transaminase only. Carbutamide at 10^{-1}M produced approximately 25% inhibition of both enzymes. Isoniazid was the most effective transaminase inhibitor and when used at a concentration of 10^{-1}M the aspartic acid/glutamic acid reaction was inhibited by 50% and the valine/glutamic acid transaminase by 85%.

Complete inhibition of overnight growth of cultures of *E. coli* was obtained with 5×10^{-5}M-propamidine, 10^{-4}M-pentamidine, 5×10^{-4}M-carbutamide and 5×10^{-3}M-isoniazid. There was thus no direct correlation between the ability of these drugs to inhibit transamination reactions and to inhibit the growth of the cultures.

Growth inhibition by 5×10^{-3}M-isoniazid and 5×10^{-5}M-propamidine was partially reversed by ten times these concentrations of glutamic acid alone or a mixture of aspartic acid, alanine and valine. Inhibition by 10^{-4}M-pentamidine required 10^{-2}M-glutamic acid or the above amino acid mixture for partial reversal. Inhibition by 5×10^{-4}M-carbutamide was reversed only by 5×10^{-2}M-glutamic acid.

I would like to thank Dr J. Gareth Morris, Department of Biochemistry, University of Oxford, for the pyridoxine-dependent mutant of *E. coli*.

* I.C.I. Research Fellow.

REFERENCES

Amos, H. & Vollmayer, E. (1957). *J. Bact.* **73**, 172.
Bornstein, J. (1957). *Nature, Lond.*, **179**, 534.
Hicks, R. M. & Cymmerman Craig, J. (1957). *Biochem. J.* **67**, 353.
Rudman, D. & Meister, A. (1953). *J. biol. Chem.* **200**, 591.

Purification of Growth Hormone from Pig Pituitaries. By J. H. OTTAWAY. (*Department of Biochemistry, University College, Gower Street, London, W.C.* 1)

Raben & Westermeyer (1951) have described the preparation of a growth-promoting principle from a glacial acetic acid extract of pig pituitaries. Although Raben (1956) has further purified this material by column chromatography on carboxymethylcellulose, there has as yet been no report of material pure enough for molecular weight determinations and end-group studies. Wilhelmi (1955) was unable to prepare pure pig growth hormone by any variant of the procedures which he had found successful for the growth hormones of the ox and other species. The preparation of a partially purified pig growth hormone by a mild extraction procedure has been investigated afresh, and the results are described below. As it has been found that one of the chief contaminants of partially purified ox growth hormone is denatured haemoglobin (Ottaway, 1958), the method has been arranged to avoid this as far as possible.

The pituitaries are placed on solid CO_2 soon after dissection, and stored at $-10°$ until required. Up to the present, the posterior lobes have not been removed. It has been found that the yield of hormone is increased if the glands are allowed to stand at room temperature for 24 hr. before extraction. During this period they are washed to remove as much blood as possible. Five hundred re-frozen pituitaries (weight approx. 125 g.) are minced finely and extracted overnight with 500 ml. distilled water at pH 6·8. This and subsequent operations are carried out in the cold. After centrifuging, the mince is re-extracted with 250 ml. of water for 1 hr. at pH 8·0. Carbon monoxide is passed through the combined extracts for 30 min., and the pH is then adjusted to 5·0. The precipitate is dissolved in 250 ml. of 0·1M-KCl at pH 9, and fractionated with ammonium sulphate. The fraction precipitating between 20 and 40% saturation

at pH 7·5 is dialysed free of salt, and the solution made 0·1 M with respect to KCl. The pH is adjusted to 5·8 and a precipitate is removed. To the supernatant is added 50 % ethanol until the ethanol concentration is 20 %. The precipitate is dialysed and freeze-dried. The purest preparation so far made (by re-fractionation with ethanol) appears to have one major impurity which may be a glycoprotein. It is free from ACTH activity.

The estimated growth hormone content of the pH 5·8 and ethanol precipitates, using the plateaued female rat as test animal, is about 12 g./kg. fresh glands, on the basis of an activity comparable with that of ox growth hormone.

I wish to acknowledge the support of the Nuffield Foundation in partially defraying the costs of this investigation.

REFERENCES

Ottaway, J. H. (1958). *Biochim. biophys. Acta*, **29**, 92.
Raben, M. S. (1956). *Proc. Soc. exp. Biol., N.Y.*, **93**, 338.
Raben, M. S. & Westermeyer, V. W. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 550.
Wilhelmi, A. E. (1955). *The Hypophyseal Growth Hormone, Nature and Actions*, p. 61. New York: McGraw Hill.

Purification and Properties of a Proteinase from *Bacillus cereus* NCTC 945. By D. N. Salter (introduced by Patricia H. Clarke). (*Department of Biochemistry, University College, Gower Street, London, W.C.* 1)

Culture filtrates of *B. cereus*, NCTC 945, were found to contain a glycyl-glycine dipeptidase and at least one proteinase acting on azocoll (Bidwell & van Heyningen, 1948). The enzyme attacking azocoll has been purified by ammonium sulphate precipitation and diethylaminoethylcellulose (DEAE-cellulose) (Peterson & Sober, 1956; Sargeant & Smith, 1958). Preliminary investigations were carried out on the ammonium sulphate precipitated enzyme.

The activity against azocoll showed a typical enzyme pH curve with a broad optimum around pH 6·8. It was also most stable when stored at this pH. Dialysis of the enzyme against 0·005 M phosphate buffer pH 6·8 resulted in decreased activity, which was almost completely restored by adding 10^{-4} M-Ca^{2+}. Activity curves were obtained by testing the enzyme at various concentrations of Ca^{2+} and phosphate in tris and phosphate buffers pH 7·4. There was an optimal Ca^{2+} concentration at 10^{-4} M in both tris and phosphate buffers and an optimum of 10^{-4} M for phosphate in tris buffer. In 0·067 M phosphate buffer pH 6·8 the enzyme was completely inhibited by 10^{-3} M-Co^{2+}, Zn^{2+} and Fe^{2+}, 70 % inhibited by Mn^{2+} and slightly inhibited by Mg^{2+}. In 0·067 M tris buffer pH 7·4, Mg^{2+} gave a similar curve to Ca^{2+} with slight activation at 10^{-4} M, while Cu^{2+} inhibited completely at 10^{-3} M. There was complete inhibition by 10^{-4} M-versene, 40 % inhibition by 2×10^{-2} M-NaCN, 25 % inhibition at 4×10^{-3} M-cysteine. Citrate gave complete inhibition at 8×10^{-2} M and 10 % at 10^{-4} M, and oxalate behaved in a similar way.

The ammonium sulphate precipitated enzyme was freeze-dried, resuspended in 0·005 M tris buffer at pH 7·4 and dialysed against the same buffer. 10 ml. of this enzyme solution, containing approximately 0·2 g. of protein, were put on a 2 g. column of DEAE-cellulose. Elution by 0·02 M tris buffer pH 7·4 gave two main protein peaks with two minor components. Most of the azocoll activity ran ahead of the main protein peak. The remaining protein was eluted by a NaCl gradient in 0·067 M tris buffer pH 7·4 and gave a broad protein band with low azocoll activity. Much of the brown pigment associated with the crude enzyme was eluted in fractions with no enzyme activity. The specific activity of the azocoll enzyme in the early fractions was about 100 times that in the original culture filtrate. Since the capacity of DEAE-cellulose is high, this could be a useful means of preparing purified enzyme, using a larger column.

REFERENCES

Bidwell, E. & van Heyningen, W. E. (1948). *Biochem. J.* **42**, 140.
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
Sargeant, K. & Smith, H. (1958). *Biochem. J.* **69**, 55P.

Hypersensitivity of the Visual Cell to Iodoacetate. By C. N. Graymore (introduced by Margaret Kerly). (*Department of Pathology, Institute of Ophthalmology, University of London, London, W.C.* 1)

It has been shown repeatedly that intravenous injection of a just sublethal dose of sodium iodoacetate selectively destroys the visual cells in the cat, monkey, rabbit (Schubert & Bornschein, 1951; Noell, 1952) and rat (Graymore & Tansley, 1959*a*). Noell (1952), on the basis of the differing effects of anoxia and iodoacetate on the electroretinogram of the rabbit, suggested that iodoacetate was acting on some mechanism which was not oxygen dependent; a predominantly glycolytic form of metabolism would account for hypersensitivity to an established glycolytic poison.

Partial support for this concept was gained from the observation that the glycolytic activity of the rat retina is doubled at the time of visual cell differentiation, and that subsequent loss of these cells, whether induced chemically by iodoacetate, or as the result of an hereditary degeneration, leads to a fall in retinal glycolysis to the immature value (Graymore, 1959; Graymore & Tansley, 1959*b*; Graymore, Tansley & Kerly, 1959). This suggested that the visual cells make a major contribution to the high glycolytic rate of the retina, and it was shown further that this glycolysis was drastically inhibited within 10 min. of treatment with iodoacetate.

Measurements of the respiratory activity of the rat retina during development, however, show that the glycolytic increase is accompanied by a proportionate increase in respiration, suggesting that the visual cells are characterized by having both a high glycolytic and a high respiratory capacity, a concept which is endorsed by the metabolic pattern seen during the development of animals having an hereditary retinal degeneration in which the visual cells are destroyed. Although preliminary investigations indicate that a dose of iodoacetate sufficient to inhibit glycolysis sharply and produce retinal damage has relatively little effect on retinal respiratory activity, the above evidence argues against the hypothesis that the selective action of iodoacetate reflects a predominantly glycolytic form of metabolism in the visual cells, and presents the idea that the overall high energy requirements of these cells might provide a clue to their hypersensitivity, even though the primary block may still prove to be glycolytic.

REFERENCES

Graymore, C. N. (1959). *Brit. J. Ophthal.* **43**, 34.
Graymore, C. N. & Tansley, K. (1959*a*). *Brit. J. Ophthal.* (in the Press).
Graymore, C. N. & Tansley, K. (1959*b*). *Brit. J. Ophthal.* (in the Press).
Graymore, C. N., Tansley, K. & Kerly, M. (1959). *Biochem. J.* (in the Press).
Noell, W. K. (1952). *J. cell comp. Physiol.* **40**, 25.
Schubert, G. & Bornschein, H. (1951). *Experientia*, **7**, 461.

Amino Acid Incorporation into Proteins of Isolated Rat-Liver Mitochondria. By P. J. Reis, J. L. Coote and T. S. Work. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Mitochondria were isolated from 0·3 M-sucrose homogenates of rat liver by differential centrifugation, and washed repeatedly to remove contaminating nuclei and microsomes. When these particles were incubated at 30°–37° with ^{14}C-amino acids under suitable conditions, there was incorporation of amino acids into the mitochondrial protein (precipitated with trichloroacetic acid and extracted to remove nucleic acids and lipids). In the presence of cell sap, buffer (pH 7·5), succinate, Mg^{2+}, K^+, versene, nicotinamide and sucrose incorporation could be maintained for up to one hour. Dinitrophenol, KCN, or anaerobiosis inhibited incorporation almost completely. Ribonuclease was without effect or produced slight stimulation; this is in marked contrast to microsome incorporation systems. Boiled (deproteinized) cell sap was as effective as fresh cell sap in promoting incorporation, indicating no requirement for amino acid activating enzymes. The cell sap could not be entirely replaced by a synthetic mixture of nucleotides and amino acids.

No amino acid activating enzymes could be demonstrated in suspensions of intact or disrupted mitochondria; extracts of acetone powders, prepared from mitochondria, promoted an amino acid dependent exchange between adenosine triphosphate and radioactive pyrophosphate. It seems likely, therefore, that some amino acid activating enzymes are present within liver mitochondria.

Rat liver catalase was purified by chromatography on diethylaminoethylcellulose and used to immunize rabbits. The rabbit antiserum specifically precipitated rat liver catalase and was used to isolate catalase from mitochondria. Mitochondria, which had been incubated with ^{14}C-amino acids under appropriate conditions, were shaken with aqueous butanol to release catalase. In three incubations, the enzyme, specifically precipitated with anticatalase, was radioactive. In several other experiments, the insoluble mitochondrial proteins were equally radioactive but the catalase was not radioactive. The cause of this discrepancy has not, so far, been established.

The results are consistent with the assumption that isolated rat-liver mitochondria can synthesize protein but meantime it is uncertain whether they can synthesize catalase (cf. McLean, Cohn, Brandt & Simpson, 1958).

REFERENCE

McLean, J. R., Cohn, G. L., Brandt, I. K. & Simpson, M. V. (1958). *J. biol. Chem.* **233**, 657.

DEMONSTRATION

An Apparatus for Incubating Tissue Slices *in vitro*. By J. H. Ottaway, A. K. Huggins and J. Martin. (*Department of Biochemistry, University College, Gower Street, London, W.C.* 1)

The apparatus described has been developed primarily for incubating rat diaphragm strips *in vitro*. It is designed to avoid the disadvantage of the Dubnoff type of shaker, which is that strips from several animals must be pooled before the incubation can begin; this introduces undesirable differences in the time interval between dissection of the muscle and the start of the incubation. The present apparatus can be adapted for the study of other tissue slices; it is shaken in a conventional Warburg bath.

It consists of a rectangular box, $3\frac{1}{2}$ in. $\times$ $2\frac{1}{2}$ in. $\times$ $1\frac{1}{2}$ in., made of $\frac{3}{16}$ in. Perspex sheet, with a detachable, flanged, watertight lid. Through the lid pass Perspex tubes used for gassing, which can be closed off by means of screw clips on rubber connexions. A Perspex tray drilled with a series of holes fits inside the box, and small glass beakers (1 cm. diameter $\times$ 2 cm. high) rest in the holes. The number of beakers can be varied as required, but it is desirable to make provision for one containing medium alone, to check that evaporation does not occur. The lid is greased and held in place by spring clips at each end. For shaking, the box is attached to a metal support by means of metal slides, as used for Warburg manometers, and a retaining spring, and the support is fixed to the bath by a similar slide.

Approximately 1 l. of gas is passed through the box in 5 min. to ensure a uniform atmosphere inside; the gas inlets and outlet have been arranged to distribute the gas as evenly as possible. It is necessary to place strips of moistened Cellosene wadding round the walls of the box, and to presaturate the gas with water vapour.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **383rd Meeting** *of the Society was held at the National Institute for Research in Dairying (University of Reading), Shinfield, Berks, on Friday, 22 May* 1959, *when the following papers were read:*

Changes in Erythrocyte Chemistry Accompanying Temporary Pectoral Muscle Lesions in Young Fowls. By D. J. Bell and W. G. Siller. (*Sections of Biochemistry and Pathology, A.R.C. Poultry Research Centre, Edinburgh* 9)

It was found that intramuscular injection of certain substances (ACTH in 16% gelatin; sterile 16% bacteriological gelatin; cortisone acetate suspended in aqueous sodium carboxymethylcellulose; the latter solution without cortisone acetate) frequently, though not invariably, produced macroscopic lesions in a limited portion of the muscle. Injections (0·1 ml./100 g. body weight) were made on three successive days and the muscles examined post mortem on the fourth day. Histologically the lesions were all similar (those induced by gelatin seemed to be most severe) and showed principally Zenker's degeneration of the muscle fibres, sarcolemmal proliferation, oedema, heterophil leucocytes and sometimes lymphoid hyperplasia. The sarcolemmal proliferation may be an indication of muscle fibre regeneration.

Compared with the level immediately before injection, the ninhydrin nitrogen of the erythrocytes (assessed by the method of Bell, McIndoe & Gross, 1959, and by direct analysis of cells washed at 0°) showed appreciable changes being elevated in animals with macroscopic lesions after ACTH in gelatin or gelatin alone. Lesions from carboxymethylcellulose solution were accompanied by a fall in erythrocyte ninhydrin nitrogen; cortisone acetate suspension gave smaller and variable changes. Controls (no injections, or saline injections) did not show such changes neither did the animals in which no macroscopic lesions were found and where histology showed recovery from inflammation and degenerative changes.

In some experiments the blood observations were continued until the wounds would be expected to have healed; in these birds the ninhydrin nitrogen returned close to the pre-injection levels.

The erythrocyte non-protein nitrogen of each animal remained remarkably constant throughout so that changes in the 'residual nitrogen' of this fraction were also observed in the birds with muscle lesions. These findings supplement those of Bell *et al.* (1959) in favour of the idea that the erythrocytes form part of the fowl's nitrogen pool, and are affected by the nitrogen requirements of the extravascular tissues. A fall in the volume occupied by the erythrocyte was observed in the blood of the affected animals.

REFERENCE

Bell, D. J., McIndoe, W. M. & Gross, D. (1959). *Biochem. J.* **71**, 355.

Physical and Chemical Properties of Mucoids from Bovine Cervical Mucin. By R. A. Gibbons. (*National Institute for Research in Dairying, Shinfield, nr. Reading*)

Mucin from the cervix of the uterus of mammals shows changes in physical properties during the oestrous cycle (Scott-Blair, Folley, Malpress & Coppen, 1941). Pooled mucin from cows in oestrus and from pregnant animals has been fractionated by preparative ultracentrifugation and fractional precipitation from solution in strong aqueous $CaCl_2$ to yield specimens of mucoid material which are homogeneous in the ultracentrifuge and which largely retain, in aqueous solution, the physical properties of the native secretion. It has been found essential, if these physical properties are to be conserved, to avoid violent physical treatment such as shaking or stirring, and to avoid drying the material until the final stage.

The materials isolated are mucoids of the blood group substance type containing fucose, galactose, glucosamine and galactosamine, and having amino acid analyses very similar to those given by Carsten & Kabat (1956). They contain also rather large amounts of sialic acid, the pregnancy mucoid moreover contains more (17·5%) than the oestrous mucoid (13·8%). Experiments designed to reveal the presence of sialoprotein have been unsuccessful, and it is concluded that the sialic acid is an integral part of the molecule as isolated. Uronic acid and

ester sulphate are not detectable even in the crude material, but a mannose-containing substance, which is eliminated during preparative ultracentrifugation, has been observed.

The flow birefringence of these materials have shown certain anomalies very similar to those reported by Tsvetkov (1955) and Frisman (1958) for polystyrene. A study of the birefringence and orientation angle curves shows that these materials are most probably, like polystyrene, flexible molecules of the 'random-coil' configuration. Using this result the molecular weights of both specimens have been determined from intrinsic viscosities and sedimentation coefficients, and found to be, for both materials, in the region of 4×10^6. The differences in intrinsic viscosities and sedimentation constants of the two mucoids indicate a higher expansion factor for the oestrous mucoid than for the pregnancy mucoid (Flory, 1953); flow birefringence data support this conclusion. The differences in physical properties of the native secretions can probably be traced to this circumstance.

REFERENCES

Carsten, M. E. & Kabat, E. A. (1956). *J. Amer. chem. Soc.* **78**, 3083.
Flory, P. J. (1953). *Principles of Polymer Chemistry.* Cornell University Press.
Frisman, E. V. (1958). *Dokl. Akad. Nauk, SSSR*, **118**, 72.
Scott-Blair, G. W., Folley, S. J., Malpress, F. H. & Coppen, F. M. V. (1941). *Biochem. J.* **36**, 1039.
Tsvetkov, V. N. (1955). *Chem. Listy*, **49**, 1419.

Inhibition of β-Glucuronidase by Glucosaccharo-1→4-Lactone. By E. Boyland, J. H. Davies and K. Williams. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W. 3, and the Royal Marsden Hospital, Fulham Road, London, S.W. 3*)

Levvy (1952) showed that glucosaccharo-1 → 4-lactone is a potent inhibitor of β-glucuronidase. The activity of mammalian β-glucuronidase in the region of its optimum pH (4·5–5·5) is reduced by about 50% by μM-glucosaccharo-1 → 4-lactone. If glucosaccharo-1 → 4-lactone is administered orally in doses of the order of 4 g. per day then sufficient is excreted in the urine to reduce the β-glucuronidase activity of the urine by 75% when this is measured at the optimum pH (Boyland, Wallace & Williams, 1957).

When the relation of activity to pH of the urine of subjects treated with saccharo-1 → 4-lactone was studied the inhibition was found to be much less in neutral solution than at pH 5. The activity of the enzyme itself at pH 7·0 is only 10% of that at the optimum pH.

Using a preparation of β-glucuronidase from normal human urine incubated with $3{\cdot}3 \times 10^{-4}$M phenolphthalein glucosiduronate the inhibition by 10^{-5}M glucosaccharo-1 → 4-lactone was 73% at pH 4·5, 72% at pH 5·0 and at pH 5·5, 53% at pH 6·0, 30% at pH 6·5 and 21% at pH 7·0. The p*K* of the carboxyl group of glucosaccharo-1 → 4-lactone is in the region of 3·3.

REFERENCES

Boyland, E., Wallace, D. M. & Williams, D. C. (1957). *Brit. J. Cancer*, **11**, 578.
Levvy, G. A. (1952). *Biochem. J.* **52**, 464.

Adenosine Deaminase—a Constitutive Enzyme of the Developing White Leghorn Chick Embryo. By D. E. Palmer and J. B. Solomon. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W. 3*)

The substrate-induced adaptation of adenosine deaminase activity by the chick embryo has been reported by Gordon & Roder (1953). After we had failed to obtain any substrate-induced adaptation of either lactic dehydrogenase or glutaminase in chick embryos we studied the adaptation of adenosine deaminase over a wider range of development than Gordon & Roder. Adenosine deaminase activity was assayed by both a spectrophotometric method based on the disappearance of adenosine and the Conway method for the measurement of the ammonia evolved. When 1- to 8-day-old embryos were injected with 20 or 25 mg. adenosine no statistically significant increases of adenosine deaminase activity of whole chick embryo or yolk-sac homogenates were observed at 24 hr. after injection. Neither single nor multiple injections of adenosine produced any significant

increases in liver adenosine deaminase activity during the period of development from 7 days of incubation to 20 weeks after hatching. In untreated embryos the level of adenosine deaminase activity remains constant in the homogenates of whole chick embryos (7·7 units) and yolk sacs (9·1 units) during 2–14 days of incubation, and in homogenates of livers (8·6 units) from 7 days of incubation to 20 weeks post-hatching (1 unit = 1 μg. adenosine deaminated/30 min./mg. of protein).

REFERENCE

Gordon, M. W. & Roder, M. (1953). *J. biol. Chem.* **200**, 859.

Some Components of Bovine Nasal Cartilage. By JEROME ROTSTEIN and MAXWELL SCHUBERT. (*The Rheumatic Diseases Unit, Northern General Hospital, Edinburgh, and The Study Group on Rheumatic Diseases, College of Medicine, New York University, New York*)

Malawista & Schubert (1958) evolved a rapid and efficient way of dissolving the chondromucoprotein portion of bovine nasal cartilage, which accounted for 42% of the total product. A method of dissolving the remaining non-chondromucoprotein portion through the use of superheating under pressure followed by tryptic digestion has now been developed. After dialysis, which removes 60% of the dissolved product, the anionic polysaccharide is first precipitated with hexammine cobaltic chloride and from this solution a fraction (F) rich in neutral polysaccharides subsequently is precipitated with ethanol.

The oxidation by periodic acid of F and of chondroitin sulphate has been compared. Within 20 min. the oxidation of F is virtually complete, whereas that of chondroitin sulphate is insignificant. After 24 hr. the oxidation of chondroitin sulphate is still not great. These results suggest that in the P.A.S. reaction, as used in the standard histological technique, it is the polysaccharide that is stained.

F is hydrolysed in 4N-HCl at 100° for 6 hr., made up to 0·1N-HCl and passed through a Dowex 50, mesh 100–200, resin column, which previously has been equilibrated with 0·1N-HCl. Then the column is washed with 0·1–1N-HCl. The 0·1–0·3N-HCl eluates contain mainly neutral sugars, a hexosamine and a hexuronic acid, while the 0·5–1N-HCl eluates are mainly amino acids. The 0·1–0·3N-HCl eluates have been chromatographed in three solvent systems and the chromatographs stained with three different stains. Five neutral sugars (mannose, galactose, glucose, fucose and ribose), one hexosamine (glucosamine) and one hexuronic acid (glucuronic acid in its lactone form) are identifiable.

REFERENCE

Malawista, I. & Schubert, M. (1958). *J. biol. Chem.* **230**, 535.

A Study of Butter Phospholipid Fractions. By J. D. BILLIMORIA and R. G. CURTIS. (*Westminster Medical School, London, S.W.* 1)

Addition of whole butter was found to diminish the Russel Viper Venom (R.V.V.) clotting-time of plasma by about 70%. The substances responsible for the activity were the phospholipids in butter, which were found to be bound as phospholipoprotein (Billimoria, Maclagan & Lockey, 1957). The proteins were sedimented from melted butter, and the lipids extracted from these were subjected to chromatography on a silicic acid column, eluted with a continuous linear gradient of methanol in chloroform. The progress of fractionation was followed by determinations of phosphorus, nitrogen, sugar, and R.V.V. clotting-times as modified (Maclagan, Billimoria & Curtis, 1958). Nine major peaks were thus detected. Paper chromatography (Lea, Rhodes & Stoll, 1955; Marinetti, Erbland & Kocken, 1957) of the peak components and their hydrolysis products has been carried out, and some preliminary conclusions have been reached concerning the chemical composition of these substances.

The peaks eluted from the column with increasing methanol concentration appear to contain (1) phosphatidic acid, (2) unidentified, (3) mainly phosphatidylserine, (4) a phosphatidylserine glycoside, (5) phosphatidylethanolamine, phosphatidylserine and an unidentified compound 'X', (6) a choline and phosphorus containing glycoside N:P:sugar (1:1:1), (7) a choline and phosphorus containing oligosaccharide with a very high sugar content, (8) lecithin, (9) sphingomyelin.

The lipids 3, 4, 5 and 7 were all very active in the R.V.V. test on human plasma, the unidentified component 'X' being the most active, and all of them were more active than synthetic dimyristoylphosphatidylethanolamine. Component 'X' is well

separated from phosphatidylethanolamine (P.E.) and can now be clearly distinguished from it. It is detected on a silicic acid paper chromatogram with Rhodamine 6G but gives negative tests with the usual ninhydrin and phosphomolybdic acid reagents.

Previous work on egg phospholipids (Poole & Robinson, 1956) has indicated the importance of P.E. as a thromboplastic agent in blood coagulation but it appears from our results that a number of more active compounds are present in butter.

REFERENCES

Billimoria, J. D., Maclagan, N. F. & Lockey, E. (1957). *Proc. Roy. Soc. Med.* **50**, 623.
Lea, C. H., Rhodes, D. N. & Stoll, R. D. (1955). *Biochem. J.* **60**, 353.
Maclagan, N. F., Billimoria, J. D. & Curtis, C. (1958). *Lancet*, ii, 865.
Marinetti, G. V., Erbland, J. & Kocken, J. (1957). *Fed. Proc.* **16**, 837.
Poole, J. C. F. & Robinson, D. S. (1956). *Quart. J. exp. Physiol.* **41**, 295.

The Metabolic Abnormality of Hartnup Disease. By M. D. Milne, M. A. Crawford, C. B. Girão and Lavinia Loughridge (introduced by C. E. Dalgliesh). (*Department of Medicine, Postgraduate Medical School, Ducane Road, London, W.* 12)

The Hartnup disease (Baron, Dent, Harris, Hart & Jepson, 1956) is a recessive hereditary metabolic disorder characterized by pellagra, attacks of cerebellar ataxia, a generalized amino aciduria, and excessive excretion of indican. Increased excretion of indolyl-3-acetic acid and of indolylacetylglutamine is not so constant as was previously thought. Studies have been made of the biochemical effects of ingestion of L- and DL-tryptophan, 70 mg./kg. body weight, in two cases of Hartnup disease, and in normal subjects. We have found that the excretion of indoleacetic acid is influenced by urinary pH, and all observations were therefore made during mild metabolic alkalosis from sodium bicarbonate ingestion with urinary pH constant at 8·0.

Urinary kynurenine was maximal 2–4 hr. after tryptophan ingestion, but was less in amount in the cases of Hartnup disease than in the normals; 0·5–1·5% of ingested tryptophan appeared as kynurenine in Hartnup disease, and 3·0–7·0% in normals. The pattern of excretion of indolic acids after tryptophan was grossly abnormal in the disease. Normally, there is a sharp rise, reaching a maximum within 2 hr., followed by a logarithmic fall to normal values within 8–10 hr. Indolylacetic acid is the main indolic acid excreted after L-tryptophan, and indolyl-3-lactic acid after DL-tryptophan. The cases of Hartnup disease showed a similar abrupt rise in urinary indolic acids, but the high excretion persisted for over 24 hr. after ingestion of tryptophan. Indolylacetic acid was in greater amount than indolyl-lactic acid, and *lesser* amounts of indolic acids were excreted after the DL- than the L-amino acid.

Urinary indican behaved similarly; normal subjects showed a variable rise, followed by a logarithmic fall, whilst in Hartnup disease the high excretion persisted for over 24 hr. Indolylacrylylglycine acid and bis-indolyl-indoxyl (Holmes-Siedle & Saunders, 1957) were shown to be present in urinary chromatograms by the method of Asatoor & Dalgliesh (1956). Since maximal excretion occurred 12 hr. after ingestion, these compounds are probably produced by bacterial metabolism of tryptophan.

The results suggest that there is a defective transport of both L- and D-tryptophan in Hartnup disease. The defect involves the cells of the proximal renal tubules and of the jejunum, and possibly also the liver cells. An abnormally low fraction of ingested tryptophan is oxidized to formylkynurenine by tryptophan peroxidase in the disease. There is no proof of an actual deficiency of the enzyme; the defect might equally well be due to a reduced contact of the enzyme and its substrate.

REFERENCES

Asatoor, A. & Dalgliesh, C. E. (1956). *J. chem. Soc.* p. 2291.
Baron, D. N., Dent, C. E., Harris, H., Hart, E. W. & Jepson, J. B. (1956). *Lancet*, ii, 421.
Holmes-Siedle, A. G. & Saunders, B. C. (1957). *Chem. & Ind.* p. 265.

The Relation between Amino Acid Activation and Cell-Sap RNA Coupling. By H. Gutfreund. (*National Institute for Research in Dairying, Shinfield, nr. Reading*)

We have reported that in preparations from guinea-pig mammary gland activation of a number of commonly occurring amino acids could not be detected, while they are coupled into cell-sap RNA in satisfactory proportions (Fraser & Gutfreund, 1958; Fraser, Shimizu & Gutfreund, 1959). Hoagland (1958) suggested that the lack of correlation between the two phenomena is due to stability differences of the twenty activating enzymes.

Considerable improvement in our techniques for the assay of activation of individual amino acids by their stimulation of pyrophosphate exchange into ATP, has enabled us to examine with greater precision identical preparations from the pH 5 fraction, containing activating enzymes and cell sap RNA, for both RNA coupling activity and pyrophosphate exchange stimulated by amino acids. Preparations treated in a variety of ways retained full activity for leucine and glycine coupling and yet no stimulation of pyrophosphate exchange could be detected. It has been reported that in some systems certain amino acids stimulated orthophosphate exchange into ATP or that exchange into GTP might occur. We could not detect any evidence for either of these exchange reactions in preparations from guinea-pig mammary glands.

There is overwhelming evidence that the amino acid–RNA compounds discovered by Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958) are important intermediates of protein biosynthesis. The requirement for ATP, as well as general energetic considerations, clearly indicate that activation of all amino acids occurs prior to their combination with RNA. We have therefore tried to discover which of the following two reactions is rate determining for the coupling reaction.

(1) Amino acid + ATP + enzyme $\rightleftharpoons$ E ~ AMP ~ amino acid + pyrophosphate,

(2) E ~ AMP ~ amino acid + RNA $\rightleftharpoons$ amino acid ~ RNA + AMP + enzyme.

Reaction (1) is insensitive to ribonuclease while reaction (2) is completely inhibited by it. The overall rate of tyrosine coupling to RNA is unchanged by addition of purified tyrosine-activating enzyme. Reaction (2) is inhibited by pyrophosphate but it is not reversed by such an addition. From these results and considerations we conclude that reaction (2) is rate determining for amino acid-RNA coupling and that low concentrations of E ~ AMP ~ amino acid, which cannot be detected by the measurement of pyrophosphate exchange, are sufficient to provide for the observed rate of reaction (2). The different characteristics of the two reactions allowed us to design procedures to determine their rates individually in unfractionated materials.

REFERENCES

Fraser, M. J. & Gutfreund, H. (1958). *Proc. Roy. Soc.* B, **149**, 392.

Fraser, M. J., Shimizu, H. & Gutfreund, H. (1959). *Biochem. J.* **72**, 141.

Hoagland, M. B. (1958). *Proc. Roy. Soc.* B, **149**, 401.

Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht, L. I. & Zamecnik, P. C. (1958). *J. biol. Chem.* **231**, 241.

Some Properties of Guinea-Pig Mammary Gland Mitochondria. By E. A. Jones and H. Gutfreund. (*National Institute for Research in Dairying, Shinfield, nr. Reading*)

We wish to report some metabolic studies on guinea-pig mammary gland mitochondria. These experiments were undertaken to enable us to correlate the oxidation of various substrates and the uptake and exchange of phosphate with the energy requirements of protein and fatty acid synthesis in mitochondria and whole cells of mammary tissue. Mammary glands from most species contain a high proportion of connective tissue and are therefore difficult to homogenize; this factor and the presence of high concentrations of calcium are probably the reasons why no satisfactory results have previously been reported from studies of oxidative phosphorylation by mammary gland mitochondria. Guinea-pig mammary tissue can be homogenized easily with a Potter homogenizer. When mitochondria are prepared from this tissue by differential centrifugation and are washed in a EDTA sucrose medium a system is obtained which gives satisfactory results if the Mg^{2+} ion concentration of the reaction mixture is not lower than 0·02 M.

With α-oxoglutarate in the presence of malonate P/O ratios of 3·6 were obtained, while with succinate values of only 1·1 have been obtained so far. Respiratory control at 30° over the first 10 min. in the absence of a phosphate acceptor is demonstrated by a more than eightfold increase in oxidation of substrate in the presence of phosphate acceptor.

In agreement with the elegant demonstration of protein synthesis in heart muscle mitochondria by Bates, Craddock & Simpson (1958), we have obtained good incorporation of [^{14}C]amino acids into the protein of guinea-pig mammary gland mitochondria. We studied the fate in mitochondria of pyrophosphate supposedly liberated by amino acid and acetate activation. We analysed for the stimulation by amino acids and acetate of [^{32}P]-phosphate incorporation into the β and γ phosphate of ATP on addition of ^{32}P-labelled ortho- or pyrophosphate. As is to be expected (Cooper & Lehninger, 1957; Boyer, Luchsinger & Falcone, 1956) orthophosphate was incorporated into ATP

under respiratory control and during oxidation of substrate. We found no significant incorporation or hydrolysis of pyrophosphate in the presence or absence of amino acids. Acetate stimulated pyrophosphate incorporation even under non-phosphorylating conditions.

REFERENCES

Bates, H. M., Craddock, V. M. & Simpson, M. V. (1958). *J. Amer. chem. Soc.* **80**, 1000.
Boyer, P. D., Luchsinger, W. W. & Falcone, A. B. (1956). *J. biol. Chem.* **223**, 405.
Cooper, C. & Lehninger, A. L. (1957). *J. biol. Chem.* **224**, 561.

Observations on 'Cage Layer Fatigue' (CLF) in Hens. By D. J. Bell, W. G. Siller and J. G. Campbell. (*Sections of Biochemistry and Pathology, and the B.E.C.C. Unit, A.R.C. Poultry Research Centre, Edinburgh* 9)

Shortly after onset of lay, cases of a severe form of CLF occurred among fully productive caged hens laying normal shelled eggs; full sisters in pens receiving identical food were unaffected. Two forms of CLF were noted, characterized by (*a*) extreme leg weakness sometimes followed, on hand-feeding, by slow but complete recovery, and (*b*) sudden death, in apparent health, shortly after laying.

In both (*a*) and (*b*) the bones were extensively decalcified and flexible, especially the sternum, ribs, skull and sometimes the pelvis; decalcification of the long bones was not so obvious. In type (*b*) the hens had died in full lay without obvious visceral abnormalities beyond generalized venous congestion and systolic heart-failure. Histologically, the bone changes differed from those typical of osteomalacia, osteoporosis and rickets and showed two characteristics: (1) marked osteoclastic activity, and (2) thinning of both the compact and spongy components.

No abnormal values were found for plasma Ca (diffusible and non-diffusible), inorganic-P and Mg (the latter kindly analysed by Dr I. MacIntyre, Postgraduate Medical School). The plasma alkaline phosphatase activity (APA) (hydrolysis of 4-nitrophenylphosphate; pH 10; 37°) was, however, extremely high in cases of type (*a*) and in one or two of (*b*) when it was possible to sample blood *ante mortem*. Bromosulphthalein liver-clearance tests (Campbell, 1957) indicated a state of hyperoestrogenization in several (*a*) cases as long as a month after the first collapse.

The APA activity of six caged hens was followed into onset of lay and subsequently. Initially, a period of intensive laying was accompanied by greatly raised APA, but in subsequent bursts of heavy production progressively smaller rises in APA were found, suggesting the establishment of homeostasis.

Skeletal calcium withdrawal accompanies egg-shell formation and would appear to be associated, at least during adaptation from the non-laying state to one of heavy production, by elevated APA. Oestrogenization of birds is known to cause skeletal decalcification; it seems possible that hyperparathyroidism may be related to hyperactivity of oestrogen-secreting tissues. CLF which so far has not been related to diet may be caused by hormone imbalance coupled with lack of exercise. The leg weakness may not be caused by skeletal defects but to muscle dysfunction due to ionic imbalance.

REFERENCE

Campbell, J. G. (1957). *J. Endocrin.* **15**, 339.

Synthesis of Cell Constituents from Acetate by *Escherichia coli*. By H. L. Kornberg, P. J. R. Phizackerley and J. R. Sadler. (*M.R.C. Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford*)

The tricarboxylic acid cycle has been shown (Gilvarg & Davis, 1956) to be the chief metabolic route for acetate oxidation and for the provision of carbon skeletons of cell constituents in *Escherichia coli*, strain W (ATCC 9637). When this organism grows on acetate as sole carbon source, ancillary reactions must occur which lead from acetate to intermediates of the cycle. Three such routes have been postulated to occur in *E. coli*: (*a*) the direct formation of succinate from acetate (Glasky & Rafelson, 1957; Glasky, Eichholz & Rafelson, 1958); (*b*) formation of a C_3-compound from acetate and carbon dioxide which, by further carboxylation, yields C_4-dicarboxylic acids (Roberts *et al.* 1955); (*c*) the glyoxylate cycle (Kornberg & Krebs, 1957; Kornberg & Madsen, 1958).

Cells growing rapidly on acetate were incubated for brief periods (4 sec.–10 min.) with [1-^{14}C]-acetate. The initial incorporation of radio-isotope indicated that acetate entered the tricarboxylic

acid cycle at two sites, to form citrate at one and malate at the other. Succinate did not acquire label at the earliest times, which argued against postulate (*a*). Similarly, the quantities of isotope incorporated from $^{14}CO_2$ were only about 10% of those incorporated from [1-^{14}C]acetate, which argued against postulate (*b*). Crude cell extracts incubated with ATP, coenzyme A, $MgCl_2$, glutathione and [1-^{14}C]acetate, catalysed the formation of labelled citrate from oxaloacetate, and of labelled malate from either *iso*citrate or glyoxylate, which confirms the presence of *iso*citratase (Olson, 1954; Smith & Gunsalus, 1954), and of malate synthetase (Wong & Ajl, 1956). Quantitative assay of these enzymes (Dixon & Kornberg, 1959) showed that such crude cell extracts were capable of forming (per mg. of soluble protein per hr.) 6·6 μmoles of glyoxylate from *iso*citrate, 5·6 μmoles of malate from acetyl coenzyme A and glyoxylate, and 22 μmoles of citrate from acetyl coenzyme A and oxaloacetate. It can be calculated that these enzymic activities are sufficient to account for the observed rates of protein synthesis during growth on acetate. Partially purified extracts catalysed the stoicheiometric conversion of acetyl coenzyme A to malate in the presence of *iso*citrate. These results show that *E. coli*, strain W, growing on acetate, utilize acetate via the tricarboxylic acid and glyoxylate cycles, and indicate that other routes of acetate utilization, such as postulated under (*a*) and (*b*) above, are of quantitatively negligible significance.

REFERENCES

Dixon, G. H. & Kornberg, H. L. (1959). *Biochem. J.* **72**, 3P.

Gilvarg, C. & Davis, B. D. (1956). *J. biol. Chem.* **222**, 307.

Glasky, A. J. & Rafelson, M. E. (1957). *Fed. Proc.* **16**, 186.

Glasky, A. J., Eichholz, A. & Rafelson, M. E. (1958). *Fed. Proc.* **17**, 230.

Kornberg, H. L. & Krebs, H. A. (1957). *Nature, Lond.*, **179**, 988.

Kornberg, H. L. & Madsen, N. B. (1958). *Biochem. J.* **68**, 549.

Olson, J. A. (1954). *Nature, Lond.*, **194**, 695.

Smith, R. A. & Gunsalus, I. C. (1954). *J. Amer. chem. Soc.* **76**, 5002.

Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Studies of Biosynthesis in* Escherichia coli. Washington: Carnegie Institution of Washington.

Wong, D. T. O. & Ajl, S. J. (1956). *J. Amer. chem. Soc.* **78**, 3230.

Influence of Growth Substrates on Levels of Glyoxylate Cycle Enzymes in *Pseudomonas ovalis* Chester. By H. L. Kornberg and Patricia Lund. (*M.R.C. Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford*)

The three key enzymes of the glyoxylate cycle (Kornberg & Krebs, 1957; Kornberg & Madsen, 1958) are (i) *iso*citratase (Olson, 1954), (ii) malate synthetase (Wong & Ajl, 1956), and (iii) 'condensing enzyme' (Ochoa, Stern & Schneider, 1951), catalysing the reactions:

(i) *iso*citrate $\rightleftharpoons$ succinate + glyoxylate,
(ii) acetyl coenzyme A + glyoxylate $\rightarrow$ malate,
(iii) acetyl coenzyme A + oxaloacetate $\rightarrow$ citrate.

Previous studies (Kornberg, Gotto & Lund, 1958) have shown *iso*citratase (i) to be adaptively formed by *Pseudomonas ovalis* Chester grown on acetate. Further work indicates that the growth of this organism on substrates (e.g. butyrate, malonate, lysine, tyrosine and phenylalanine), the catabolism of which yields acetate or acetoacetate as principal products in mammalian systems, is also accompanied by *iso*citratase formation. Both *iso*citratase formation and growth were abolished or arrested by addition of chloramphenicol (50 μg./ml.) to succinate-grown cells adapting to acetate: thus *iso*citratase formation appears to be associated with *de novo* synthesis of protein.

Wong & Ajl (1957) claimed that the malate synthetase (ii) of *Escherichia coli* was also under adaptive control, and was found in high activity only in acetate-grown cells. Analysis of the levels of this enzyme and of the 'condensing enzyme' (iii) in *P. ovalis* Chester have shown these enzymes to be present under all conditions of growth in high activity, the specific activity of 'condensing enzyme' being about 2–3 times that of malate synthetase as measured by the optical assay of Dixon & Kornberg (1959). Although adaptation from growth on succinate to acetate was accompanied by a thirty-fold increase in the specific activity of *iso*citratase, no differences in those of malate synthetase or 'condensing enzyme' were detected. This suggests that *iso*citratase is the only enzyme of the glyoxylate cycle under adaptive control, and that malate synthetase is constitutive to *P. ovalis* Chester.

REFERENCES

Dixon, G. H. & Kornberg, H. L. (1959). *Biochem. J.* **72**, 3P.

Kornberg, H. L. & Krebs, H. A. (1957). *Nature, Lond.*, **179**, 988.

Kornberg, H. L. & Madsen, N. B. (1958). *Biochem. J.* **68**, 549.

Kornberg, H. L., Gotto, A. M. & Lund, P. (1958). *Nature, Lond.*, **182**, 1430.

Ochoa, S., Stern, J. R. & Schneider, M. C. (1951). *J. biol. Chem.* **193**, 691.

Olson, J. A. (1954). *Nature, Lond.*, **194**, 695.

Wong, D. T. O. & Ajl, S. J. (1956). *J. Amer. chem. Soc.* **78**, 3220.

Wong, D. T. O. & Ajl, S. J. (1957). *Science*, **126**, 1013.

The Formation of 'Terpenoid' Acids from Mevalonic Acid in Liver Enzyme Preparations and their Relation to Sterol Biosynthesis. By G. Popják, Marjorie Horning,* Nancy L. R. Bucher† (*M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W. 12*), *and* Rita H. Cornforth (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

The rat-liver enzyme system that synthesizes squalene and cholesterol from mevalonic acid consists of a soluble protein fraction and microsomes; it requires ATP, Mg^{2+}, DPNH and TPNH as coenzymes and glutathione or ascorbic acid as activator in aerobic incubations (Popják, Gosselin, Gore & Gould, 1958). The soluble enzyme fraction catalyses the phosphorylation of mevalonic acid in the presence of ATP and Mg^{2+}, but if TPNH is also added to the incubation mixtures the phosphorylated derivatives (e.g. 5-phosphomevalonic acid) disappear and acidic products accumulate (cf. Popják, 1959). These acids can be readily extracted with light petroleum (b.p. 40–60°) or with *n*-hexane from the incubation mixtures, without previous saponification, provided ethanol to a concentration of 50% (v/v) had been added to the mixture and the pH adjusted to about 3·0. Resolution of the acids by reversed-phase chromatography on columns with medicinal paraffin as stationary phase (Howard & Martin, 1950) showed that at least three groups of acids are formed from [2-^{14}C]mevalonic acid. A more detailed analysis by gas-liquid radiochromatography (Popják, Lowe, Moore, Brown & Smith, 1959) of the methyl esters of these acids revealed the formation of not less than 15 new compounds, the retention volumes of which on both Apiezon-L and ethylene glycol-adipate polyester columns differed from all recognized *n*-fatty acids. Of the thirteen substances four constitute the major products: these have relative retention volumes on the polyadipate columns of 0·31, 0·6, 3·78 and 6·0, the retention volume of methyl stearate being taken as 1·0. The substance with a relative retention volume of 0·61 is indistinguishable chromatographically from the methyl ester of all-*trans*-farnesoic acid (farnesenic acid). Maximum yields of these acids are obtained when the soluble enzyme fraction is first incubated with all the cofactors for 1 hr. under N_2 followed by an aerobic period. If, however, at the beginning of the aerobic period microsomes and ascorbic acid are added together with DPNH and TPNH, there is no increase in the yield of acids, but cholesterol and squalene accumulate instead, and some of the acidic products formed during the anaerobic period disappear. There is as yet no unequivocal evidence as to the direct conversion of the acids into squalene and sterol, but the data show that at least they are derived from the same intermediates as is squalene or cholesterol. As some of the longer chain acids still contain the carboxyl-carbon of mevalonic acid, it is thought that during polyisoprenoid synthesis from mevalonic acid in the mammalian enzyme system decarboxylation of the phosphorylated derivatives of mevalonic acid is not an obligatory step before condensation of mevalonic acid units.

* On leave from the National Heart Institute, National Institutes of Health, Bethesda, Maryland.

† On leave from The John Collins Warren Laboratories of the Huntington Memorial Hospital, Harvard University.

REFERENCES

Howard & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.

Popják, G. (1959). *Biosynthesis of Terpenes and Sterols*, p. 148. A Ciba Foundation Symposium. London: J. and A. Churchill, Ltd.

Popják, G., Gosselin, L., Gore, I. Y. & Gould, R. G. (1958). *Biochem. J.* **69**, 238.

Popják, G., Lowe, A. E., Moore, D., Brown, L. & Smith, F. A. (1959). In preparation for publication.

Liver Mevalonic Kinase. By H. R. Levy* and G. Popják. (*M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W.* 12)

Since the recognition of mevalonic acid as a key intermediate in cholesterol biosynthesis (Tavormina, Gibbs & Huff, 1956), a number of investigators have demonstrated that phosphorylation of mevalonic acid by ATP precedes its conversion to sterol (Tchen, 1957; Lynen, 1959; de Waard & Popják, 1959; cf. Popják, 1959). The enzyme catalysing this phosphorylation has been partially purified from yeast and named mevalonic kinase (Tchen, 1958).

In this laboratory, cholesterol biosynthesis from mevalonic acid has been studied in rat-liver preparations (Popják, Gosselin, Gore & Gould, 1958), but extracts of rat liver were unsuitable as a starting material for the purification of mevalonic kinase, because of too many interfering reactions in such preparations. Extracts of finely-chopped pig liver, made according to the method of Finch & Hele (to be published), and treated with protamine contained more mevalonic kinase and fewer interfering enzymes than extracts of yeast or rat liver. The enzyme was purified more than fifty-fold by ammonium sulphate fractionation and by treatment with calcium phosphate gel. Our most purified preparation catalysed the phosphorylation of 1·2 μmoles of mevalonic acid/min./mg. protein at 37°.

The reaction can be demonstrated by the identification of 5-phospho[^{14}C]mevalonate by paper chromatography, or by a spectrophotometric assay based on the measurement with pyruvic kinase and lactic dehydrogenase of the ADP formed in the reaction (Tchen, 1958; Lynen, 1959). This assay, which could be applied even to crude liver extracts, was carried out by incubating at 37° mevalonic kinase, potassium DL-mevalonate, ATP, Mg^{2+}, cysteine and NaF with an excess of phosphoenolpyruvate, DPNH, pyruvic kinase and lactic dehydrogenase and by following oxidation of DPNH at 340 mμ in a Beckman-recording spectrophotometer.

Optimum conditions for the action of mevalonic kinase were obtained in the presence of 0·66–10 mM-cysteine, 5 mM-Mg^{2+}, 7 mM-ATP, and 2 mM-(DL)-mevalonate. Cysteine was needed even in the most highly purified preparations in which, at a concentration of 0·66 mM, it caused a sixfold stimulation of activity. Glutathione, but not ascorbic acid, could replace cysteine. Concentrations of the reactants (except that of cysteine) higher than stated were inhibitory. Mn^{2+} could replace Mg^{2+} only at very low concentrations (0·3 mM); at a concentration of 3 mM, or higher, it inhibited strongly mevalonic kinase (but not pyruvic kinase).

Partially purified preparations of mevalonic kinase have been used to prepare 5-phospho[2-^{14}C]-mevalonate.

* Postdoctoral Fellow, National Cancer Institute, U.S. Public Health Service.

REFERENCES

Lynen, F. (1959). *Ciba Foundation Symposium on the Biosynthesis of Terpenes and Sterols*, p. 95. London: J. and A. Churchill, Ltd.

Popják, G. (1959). *Ciba Foundation Symposium on the Biosynthesis of Terpenes and Sterols*, p. 148. London: J. and A. Churchill, Ltd.

Popják, G., Gosselin, L., Gore, I. Y. & Gould, R. G. (1958). *Biochem. J.* **69**, 238.

Tavormina, P. A., Gibbs, M. H. & Huff, J. W. (1956). *J. Amer. chem. Soc.* **78**, 4498.

Tchen, T. T. (1957). *J. Amer. chem. Soc.* **79**, 6344.

Tchen, T. T. (1958). *J. biol. Chem.* **233**, 1100.

Waard, A. de & Popják, G. (1959). *Biochem. J.* Submitted for publication.

Ornithuric Acid Formation in Hens. By B. C. Baldwin, D. Robinson and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W.* 2)

L-Ornithuric acid (N^2N^5-dibenzoyl-L-ornithine) is a metabolite in hens of benzoic acid (Jaffe, 1877) and of metabolic precursors of benzoic acid (benzaldehyde, phenylpropionic acid and cinnamic acid; Crowdle & Sherwin, 1923). Conjugation with ornithine is apparently a general reaction of aromatic acids in hens and is analogous with glycine conjugation in mammals. The extent of ornithine conjugation, however, has not been clearly defined.

Paper chromatography of urine from hens after dosing with [^{14}C]benzoic acid revealed two radioactive spots, one corresponding to benzoyl glucuronide and the other to ornithuric acid. Spots corresponding to hippuric acid and N^2- and N^5-monobenzoylornithines were not found. A hen which received 500 mg. (28 μc) of [^{14}C]benzoic acid excreted 96% of the ^{14}C in 17 hr. Of the ^{14}C excreted 40% was present in ornithuric acid (by isotope dilution) and 39·5% in benzoyl glucuronide

(determined by isotope dilution as benzoic acid after alkaline hydrolysis of the urine). In two other experiments, 76 and 77% of the ^{14}C was excreted in 19 hr. and the ornithuric acid amounted to 25 and 43% respectively of the ^{14}C excreted. In these experiments mixed urine and faeces were analysed and bacterial decomposition was prevented by $HgCl_2$. In one experiment in which no precautions against bacterial decomposition were used, the ornithuric acid was found to be only 0·5% of the dose; however, 5·5% of N^5-monobenzoylornithine was found. Hen faeces, but not urine, contain enzymes which destroy ornithuric acid. If L-ornithuric acid is incubated at pH 7 with hen faeces it is gradually destroyed; N^5-monobenzoylornithine, but not N^2-benzoylornithine, appears in the incubation mixture. Eventually the N^5-derivative disappears. It was found that 0·1 g. of wet faeces destroyed 2 mg. of L-ornithuric acid at pH 7 within 21 hr.

Benzamide is also converted in the hen to ornithuric acid and benzoylglucuronide. Both these compounds were isolated from the urine, the ornithuric acid being characterized as the methyl ester and the glucuronide as the methyl ester and triacetyl methyl ester (cf. Baldwin, Robinson & Williams (1959)).

The work was supported by a grant from Dr Salsbury's Laboratories, Charles City, Iowa.

REFERENCES

Baldwin, B. C., Robinson, D. & Williams, R. T. (1959). *Biochem. J.* **71**, 638.
Crowdle, J. H. & Sherwin, C. P. (1923). *J. biol. Chem.* **55**, 15.
Jaffe, M. (1877). *Ber. dtsch. chem. Ges.* **10**, 1925.

Purification of a Nitro-reductase Enzyme of a *Nocardia* sp. By J. R. Villanueva.* (*M.R.C. Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Recent advances in the preparation and uses of ion-exchange agents (Peterson & Sober, 1956) for the study of proteins suggested their application to the purification of a nitro-reductase from *Nocardia* sp. Estimation of nitro-reductase activity, using *p*-dinitrobenzene as substrate, and some of the properties of the crude enzyme have been already reported (Villanueva, 1959).

Some of the classical methods of enzyme purification have been attempted, but these procedures usually gave little purification and resulted in considerable loss of enzyme activity.

An extract prepared by ultrasonication of acetone dried cells in 0·06M-phosphate buffer pH 7·6, containing 1×10^{-3}M-L-cysteine, was used as starting material. The extract was centrifuged at 4000 *g* and the supernatant fractionated by adding solid ammonium sulphate. The material precipitating between 0·45 and 0·65 saturation was treated with protamine, centrifuged and the supernatant dialysed overnight against 0·01M-phosphate buffer pH 7·6 (containing L-cysteine). The dialysed fraction was applied to a 1×18 cm. column of diethylaminoethyl cellulose, prepared according to Peterson & Sober (1956), and gradient elution carried out at 4° with NaCl (0·05–0·4M) in 0·01M-phosphate buffer pH 7·6. Fractions of 4 ml. were collected and analysed for protein (Kalckar, 1947) and enzyme activity. Fractions with more than 200-fold purification were obtained. Considerable loss of activity was evident as the purity increased. Flavins did not reactivate when added to the assay system. An organic sulphydryl compound was necessary for nitro-reductase activity, and with the highly purified fraction DPNH was 10 times more active than TPNH as hydrogen donor.

The highly purified chromatographic fractions were combined, concentrated by ammonium sulphate precipitation, dialysed and examined in an analytical ultracentrifuge. The sedimentation patterns showed a single peak.

Attempts were also made to purify the enzyme by using electrophoretic methods with starch, cellulose powder or paper strips at 1000v or less, and a wide range of buffers and pH values. Although the nitro-reductase activity appeared to be slightly displaced behind the major protein peak, no significant purification was obtained. However, by using paper strips at higher voltage (6000–8000v) in a Locarte high-voltage apparatus with 0·02M-phosphate buffer (plus L-cysteine) at pH 8, clear separation of the enzyme from the bulk of the protein was obtained although there was considerable inactivation.

* Ramsay Memorial Fellow.

REFERENCES

Kalckar, H. M. (1947). *J. biol. Chem.* **167**, 461.
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
Villanueva, J. R. (1959). *J. gen. Microbiol.*, **20**, ix.

The Occurrence of Hydroxystilbenes in *Eucalyptus*. By D. E. HATHWAY and J. W. T. SEAKINS. (*British Leather Manufacturers' Research Association, Milton Park, Egham, Surrey*)

The isolation of 3:5:4′-trihydroxystilbene and its 3β-D-glucoside from *Eucalyptus wandoo* heartwood by chromatography of the ether-soluble extractives on cellulose and polyamide columns (Hathway & Seakins, 1959), suggests that these hydroxystilbenes may behave as taxonomic tracers in the genus *Eucalyptus*. Erdtman (1958) has investigated the significance of the incidence of 3:5-dihydroxy- and 3-hydroxy-5-methoxy-stilbenes in pine heartwoods to the taxonomy of the genus *Pinus*, and a similar investigation using Eucalypt heartwoods is now being made in this laboratory.

Preliminary results on the subsection Longiores of the Macrantherae (Blakely, 1934) shows that both phenols together with a third hydroxystilbene, which is a minor constituent of *E. wandoo*, are present in five out of six of the heartwoods examined. The three hydroxystilbenes are present in *E. calophylla* R.Br., *E. corymbosa* Sm. and *E. maculata* Hook. of the Corymbosa–Peltatae series and in single representatives, *E. astringens* Maiden and *E. wandoo*, of the Cornutae and Subcornutae series respectively, but they are absent from *E. saligna* Sm. of the Transversae series. The heartwoods of further species belonging to the Longiores and to other subsections of the Macrantherae need to be screened before the importance of hydroxystilbenes to taxonomy can be fully assessed.

E. sideroxylon Benth. of the section Terminales does not contain hydroxystilbenes.

The hydroxystilbenes were initially identified by their locations on two-dimensional chromatograms which were run in alternative pairs of solvent systems, by their intense blue fluorescence in ultraviolet light, by the red colour with tetrazotized benzidine and by the abnormal blue colour with vanillin. On hydrogenation, the fluorescence is quenched, and the dihydrostilbenes which are formed, undergo a characteristic shift in the N-acetic acid solvent system and give the normal vanillin reaction. In this solvent system, the R_F values of 3:5:4′-trihydroxystilbene and the corresponding dihydrostilbene are 0 and 0·50 respectively, and those of 3:5:4′-trihydroxystilbene-3β-D-glucoside and the corresponding dihydrostilbene are 0·27 and 0·70 respectively.

REFERENCES

Blakely, W. F. (1934). In *A Key to the Eucalypts*. Sydney: The Worker Trustees.

Erdtman, H. (1958). *Symposium no.* 11/9. *IVth International Congress of Biochemistry*. Wien.

Hathway, D. E. & Seakins, J. W. T. (1959). *Biochem. J.* **72**, 369.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **384th Meeting** *of the Society was held at the Beveridge Hall of the Senate House, University of London, on Tuesday,* 30 *June* 1959, *when the following papers were read:*

SYMPOSIUM ON STERIC ASPECTS OF THE CHEMISTRY AND BIOCHEMISTRY OF NATURAL PRODUCTS

Stereochemical Correlations. By W. KLYNE. (*Postgraduate Medical School, London, W.* 12)

Stereochemical correlation means the study of the relative and absolute configuration of asymmetric compounds. Since most biologically active compounds are asymmetric, knowledge of their configurations is essential both for the structural organic chemist and the biochemist.

Stereospecificity is characteristic of many (perhaps all) enzymes and therefore steric relationships are important in biochemical work at all levels—from studies of isolated pure enzymes to studies on the whole organism—and of all types, e.g. work on metabolism, biogenesis, drug action.

Much important work is in progress on the shape and large-scale structure of proteins, nucleic acids, and polysaccharides. The subsequent study of the finer details, which will be necessary for an understanding of enzyme–substrate interactions, will necessitate a full knowledge of the relative stereochemistry of the smaller molecules which act as substrates, and as building-blocks for the organic macromolecules. This has been pointed out by Theorell (1957) in a Nobel Prize address, and from the organic chemical side, two leading workers have suggested the preparation of 'imitation enzymes' as the next major goal for synthetic studies (Todd, 1956; Woodward, 1956).

Until a few years ago, all configurations were designated relative to an arbitrary standard (glyceraldehyde or alanine). It is now known, thanks to the X-ray work of Bijvoet, Peerdeman & van Bommel (1951) that, by a fortunate chance, the Fischer convention for glyceraldehyde represents its true absolute configuration.

The methods for correlating configurations have been reviewed extensively elsewhere (Mills & Klyne, 1954). Examples taken from different fields of organic chemistry and chiefly from recent literature will be used to illustrate the principal methods, viz.: (i) chemical degradation or synthesis without change at the asymmetric centre concerned; (ii) kinetic and mechanistic methods; (iii) formation of quasi-racemic compounds; (iv) optical rotation and rotatory dispersion methods; (v) X-ray crystal analysis; (iv) enzymic methods.

REFERENCES

Bijvoet, J. M., Peerdeman, A. F. & van Bommel, A. J. (1951). *Nature, Lond.*, **168**, 271.
Mills, J. A. & Klyne, W. (1954). *Progr. Stereochem.* **1**, 177.
Theorell, H. (1957). *Experientia*, **13**, 2.
Todd, A. R. (1956). In *Perspectives in Organic Chemistry*, p. 245 (ed. Todd, A. R.). New York: Interscience.
Woodward, R. B. (1956). In *Perspectives in Organic Chemistry*, p. 155 (ed. Todd, A. R.). New York: Interscience.

Steric Aspects of the Biosynthesis of Terpenes and Steroids. By D. ARIGONI. (*Laboratorium für organische Chemie, Technische Hochschule Zürich*)

According to recent developments the biosynthesis of cyclic terpenes is known to proceed through aliphatic precursors formed by head to tail or head to head condensation of biological C_5-units closely related to mevalonic acid. The latter is an optically active compound, the absolute configuration of which was recently established. Mevalonic acid is formed in a stereospecific manner by condensation and partial reduction of three units of acetyl coenzyme A.

On the route from mevalonic acid optical activity is first lost in the formation of the aliphatic terpenes, which bear no centre of asymmetry and can only exhibit geometrical isomerism around their double bonds. The formation and condensation of the biological *iso*pentane units must nevertheless be highly stereospecific as can be demonstrated by the use of radioisotopes.

A major stereochemical problem arises in the formation of the different terpenes from the aliphatic precursors, as several centres of asymmetry are newly created in the products. Two main problems need be considered here: (*a*) the steric course of the cyclization, (*b*) the stereospecificity of the rearrangements of hydrogen and carbon atoms which are often coupled with the cyclization.

An interpretation of the steric course of these biological cyclizations is possible on the basis of knowledge gained in the study of acid-catalysed cyclization *in vitro*. The cyclization is treated as a special case of electrophilic addition; its stereospecificity can be visualized by the assumption of cationic intermediates with a stable configuration. An essentially similar treatment involving the same cationic intermediates can also account for the stereospecific course of the rearrangements which occur. The stereochemistry of the products can thus be deduced unequivocally from the geometry of the double bonds and from the conformation of the cyclizing molecule. The choice of a given conformation is dictated *in vitro* by thermodynamic factors; *in vivo* this choice seems to be the main role of the enzyme system.

The extension of this scheme to the biosynthesis of the polycyclic triterpenes allows a straightforward deduction of all their stereochemical details from the geometry and conformation of the common precursor, the aliphatic hydrocarbon squalene. Chemical and biochemical evidence has recently been adduced in support of the scheme.

The situation is more confused in the field of diterpenes and sesquiterpenes, especially with the latter since severe limits seem to be imposed on any rationalization of their stereochemistry. In the light of some recent experimental work caution has to be used also in discussing possible biogenetic relationships based on a comparison of absolute stereochemistry.

Steric Aspects of Drug Action. By R. B. Barlow. (*Department of Pharmacology, University of Edinburgh*)

Present theories of the ways in which drugs act date from the work of Overton and Meyer and of Ehrlich at the beginning of this century. Overton and Meyer studied the effects of narcotics and explained their actions by supposing that they affected the cells in the central nervous system by some physicochemical process. Ehrlich's ideas, based on his work with arsenical drugs, postulated the existence of specific structures, called receptors, at which the drugs act. Both these types of explanation are still acceptable to-day but receptors are more frequently invoked than physicochemical processes in attempts to interpret the actions of drugs.

The receptor is often thought of as being an active centre on an enzyme. Knowledge of the three-dimensional structure of this active centre should help in the design of new and more active drugs. Unfortunately the identity and even the nature of the enzyme involved is usually completely unknown, and development of new drugs proceeds empirically. It is then customary to argue backwards from the structure of active compounds to infer the structure of the receptor. To be satisfactory, this process of argument demands, among other things, accurate knowledge of the structure of the drugs concerned and has in recent years benefited greatly from advances in knowledge of both absolute stereochemical configuration and the most likely conformation of flexible molecules.

Examples will be given of advances in stereochemistry which have led to developments in our understanding of the nature of receptors affected by analgesics and of those affected by acetylcholine. In conclusion reference will be made to eserine-like substances, which are known to produce their pharmacological effects by actions on a particular type of enzyme.

***cis-trans* Isomers of Retinene in Visual Processes.** By G. A. J. Pitt and R. A. Morton. (*Department of Biochemistry, University of Liverpool*)

In the last few years, much knowledge has been obtained of the isomers of retinene. Their great importance in visual processes has been revealed largely by the work, which will be discussed, of the Harvard School led by Wald and Hubbard.

Among the visual pigments most is known of the rod pigment rhodopsin. It is made up of 11-*cis*-retinene (vitamin A aldehyde) combined with a specific protein, opsin. Photochemical destruction of rhodopsin *in vitro* splits it to yield free opsin, and the stable all-*trans* isomer of retinene. In the eye this can be isomerized back to the active but less stable 11-*cis* form by an enzyme, retinene isomerase, thus completing an isomerization cycle.

When rhodopsin is broken down photochemically, it passes through a series of intermediates designated lumi-rhodopsin, meta-rhodopsin and indicator yellow, before splitting to opsin and all-*trans* retinene. The action of light on rhodopsin itself is to isomerize the 11-*cis*-retinene portion of

the molecule mainly to the all-*trans* configuration. This is the rhodopsin → lumi-rhodopsin step and is the only reaction in the breakdown of rhodopsin which is photochemical; those that follow are thermal reactions. Light can, however, reisomerize the all-*trans* retinene part of lumi-rhodopsin (or of meta-rhodopsin) back to the 11-*cis* configuration and so reform rhodopsin. It appears that visual pigments can be formed not only by the isomerization cycle mentioned above via free retinene and opsin, but also, to an extent not so far assessable, by the action of light on the early photoproducts of rhodopsin.

There is evidence that the cone pigment iodopsin, and probably other visual pigments, behave quite similarly to rhodopsin. If this is true, the direct effect of light on visual pigments *in situ* will be limited to isomerizing the retinene moiety. The switch from the 11-*cis* to the all-*trans* configuration will trigger off the complex of operations which results in the passing of an impulse up the optic nerve.

This concept and the classical earlier work on the photochemistry of visual pigments are not in conflict and the difficulties in the physiology of vision which remain are not affected by the *cis-trans* aspect.

Steric Factors in Enzyme Action: Hydrolytic Enzymes. By EDWIN C. WEBB. (*Department of Biochemistry, University of Cambridge*)

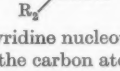
Hydrolytic enzymes provide much favourable material for a study of the stereochemical aspects of enzyme action, since so many of these enzymes are of comparatively wide specificity. Thus even if the typical substrate is a symmetrical molecule, it is often possible to introduce asymmetric groupings into this substrate without loss of activity. To be of real value, however, such studies should be carried out by measuring both V and K_m for pairs of stereoisomers, and many of the published data fall short of this ideal.

The talk will consist primarily of a survey of the available information about the action of hydrolytic enzymes on asymmetric substrates. Particular attention will be given to ali-esterases, amylases, glycosidases and peptidases. In some cases measurements of inhibition by asymmetric competitive inhibitors have also been made, and these give unambiguous information about the stereochemical requirements for combination with the active centre. This is especially true for some of the proteolytic enzymes such as chymotrypsin and papain.

The significance of these results for theories of enzyme mechanism, and in particular of the nature of the active centre, will be discussed.

Steric Factors in Enzyme Action: Oxidation–Reduction Enzymes. By E. C. SLATER. (*Laboratory of Physiological Chemistry, University of Amsterdam, The Netherlands*)

This paper will be largely devoted to the work of the school of Vennesland on the dehydrogenases which react with the pyridine nucleotides (see, for example, Vennesland, 1955, 1958). She has shown that when substrates of the general formula



are oxidized by pyridine nucleotides, the hydrogen atom attached to the carbon atom of the substrate moves to the carbon atom in position 4 of the pyridine ring, while the hydroxyl hydrogen atom reacts with the N^+ atom of the pyridine ring and dissociates as an H^+ ion. Alcohol dehydrogenase is stereospecific with respect to the carbon-bound hydrogen atom of ethanol which is transferred. All the pyridine nucleotide dehydrogenases which have been investigated are also stereospecific with respect to the two hydrogen atoms bound to the carbon atom in position 4 of the pyridine ring in the reduced pyridine nucleotide. Alcohol, lactic, malic, acetaldehyde, glyceric and dihydro-orotate dehydrogenases involve a transfer to and from one side (A) of the plane of the pyridine ring of diphosphopyridine nucleotide, while testosterone, α-glycerol phosphate, 3-phosphoglyceraldehyde, glutamic, glucose and β-hydroxybutyryl-CoA dehydrogenases, pyridine nucleotide transhydrogenase and reduced diphosphopyridine nucleotide–cytochrome *c* reductase involve the other side (B). *iso*Citric dehydrogenase operates on the A side of triphosphopyridine nucleotide, while glutamic and glucose 6-phosphate dehydrogenases and glutathione reductase operate on the B side (Vennesland, 1958).

Englard & Colowick (1956) have shown that succinic dehydrogenase (which does not react with pyridine nucleotide) is non-stereospecific with respect to the methylene hydrogen atoms of the substrate.

REFERENCES

Englard, S. & Colowick, S. P. (1956). *J. biol. Chem.* **221**, 1019.

Vennesland, B. (1955). *Disc. Faraday Soc.* no. 20, 'The Physical Chemistry of Enzymes', p. 240.

Vennesland, B. (1958). *Fed. Proc.* **17**, 1150.

Stereochemical Specificities of Some Enzymes of Nucleotide Metabolism. By G. R. BARKER.
(*Department of Chemistry, University of Manchester*)

Many of the reactions whereby simple nucleotides and polynucleotides are synthesized and broken down by tissues have been carried out in cell-free systems. Most of the enzymes concerned in these reactions are highly specific as is evident from the relatively few variations in the structures of the natural nucleotides.

The almost universal presence of residues of D-ribose and 2-deoxy-D-ribose in natural nucleotides, and the rare occurrence of these structures other than in nucleotides or their derivatives, suggest a rigid stereochemical requirement in the reactions leading to their formation. The invariable presence in natural nucleotides of the furanose configuration is a reflexion of the biosynthetic route which will be discussed.

With one exception, the nucleosidic linkage is of the β-type and this involves the question of the stereospecificity of a variety of enzymes concerned in the formation and/or fission of the *N*-riboside or *N*-deoxyriboside linkage. These include three main types: the nucleoside hydrolases, those bringing about phosphorolysis of nucleosides and nucleotides and those involving exchange reactions. In some of these reactions there is net inversion and in others no net inversion of configuration. In particular it is of interest that net inversion of configuration occurs at the anomeric position during phosphorolysis by nucleoside phosphorylase and related enzymes, whereas no net inversion results during the phosphorolysis of polysaccharides.

Some monophosphatases which hydrolyse nucleotides show some stereospecificity, but the greatest degree of selectivity is shown by enzymes catalysing the formation and fission of internucleotide linkages of the ribopolynucleotides.

Crystalline pancreatic ribonuclease exhibits a considerable degree of steric specificity in yielding only 3′-dihydrogen phosphates from pyrimidine nucleoside 2′:3′-cyclic phosphates, in attacking only 3′-alkyl hydrogen phosphates to yield appropriate 2′:3′-cyclic phosphates, and in yielding only 3′-alkyl hydrogen phosphates in the reversal of this reaction. The question will be discussed as to whether this enzyme fulfils only a degradative function or whether, in view of its specificity, it is concerned in either biosynthesis or exchange. Chemical reactions analogous to those catalysed by ribonuclease have been studied and a chemical interpretation of the steric course of the enzymic reaction has been suggested.

Examination of polymers produced by polynucleotide phosphorylases reveals that the enzymes are selective in the type of ester bond produced and this will be discussed in terms of the stereochemistry of the substrates.

ENZYMES FROM OUR CURRENT CATALOGUE—

Acid phosphatase (wheat germ)
Acylase, ex hog kidney
Alcohol dehydrogenase, ex liver
Alcohol dehydrogenase, ex yeast
Aldolase, ex muscle
D-Amino acid oxidase
Amino tripeptidase
α-Amylase
β-Amylase
Aneurine pyrophosphate HCl
Arginine decarboxylase
Aspartic decarboxylase
Bovine fibrinogen, fraction I
Bovine glycoprotein, fraction VI
Bovine haemoglobin
Bovine plasma albumin, cryst.
Bovine plasma albumin, fraction V
Bromelin
Carbonic anhydrase, purified
Carboxypeptidase, cryst.
Carnosinase
Catalase, ex beef liver
Catalase, ex liver
Cathepsin C
Cellulase (tech.)
α-Chymotrypsin
β-Chymotrypsin
γ-Chymotrypsin
Clostridium kluyveri—dry culture
Cocarboxylase
Coenzyme I, 95/100 %|
Coenzyme A, ex yeast
Collagenase, form I
Collagenase, form II
Cytochrome C, powder
Cytochrome C, solution
Deoxyribonuclease (25 % of activity of cryst.)
Deoxyribonuclease (1 × cryst.)
Diamine oxidase (hog kidney)
Diastase (ex mould organism)
Dihydro-cozymase (reduced DPN)
Egg albumin cryst.
Elastase
Enolase, ex muscle
Erepsin
Ficin (crude)
Ficin (cryst. ex fig tree latex)
Flavinadeninedinucleotide (FAD)
γ-Globulins (ex bovine, fraction II)
Glucose oxidase, pure powder
Glucose oxidase, solution
Glucose-6-phosphate
β-Glucosidase (ex almond emulsin)
β-Glucuronidase
L-Glutamic acid decarboxylase
Glutamic acid dehydrogenase, ex liver
Glyceraldehyde phosphate dehydrogenase, ex muscle
α-Glycerophosphate dehydrogenase, ex muscle
Hemicellulase
Hexokinase
Histidine decarboxylase
Histone (from calf thymus nuclei)
Hyaluronic acid
Hyaluronic acid potassium salt
Hyaluronic acid sodium salt
Hyaluronidase
Hyaluronidase inhibitor
Hydrolase mixture, ex aspergillus oryzae, powder
Invertase scales, from top fermented yeast
Lactic acid dehydrogenase, ex muscle
β-Lactoglobulin, ex cow's milk
Lipase (Steapsin)
Lipase (ex wheat germ)
Lipoxidase
Lysine decarboxylase
Lysozyme, cryst.
Malic dehydrogenase, ex heart
Mycozyme
Mylase-P
Myokinase, ex muscle
Ornithine decarboxylase
Ovalbumin, 2 × cryst.
Ovomucoid trypsin inhibitor, ex eggs
Pancreatin, triple USP
Papain
Papain, purified
Pectinase
Penicillinase
Pepsin (porcine mucosa) cryst.
Pepsin powder—1:10,000
Pepsinogen (swine)
Peptidase (hog intestinal mucosa extract)
Peroxidase
Phosphatase, alkaline, intestinal
Phosphatase, alkaline purified
Phosphatase, intestinal ex calf mucosa alkaline
Phospho-enol pyruvic acid, silver barium salt
3-Phosphoglyceric phosphokinase
Phosphorylase 'A' (cryst. susp. ex rabbit muscle)
Pigeon liver acetone powder
Pig heart powder
Pig kidney powder
Protease
Proteinase
Pyruvate kinase, ex muscle.
Rhodanese
Ribonuclease, cryst. salt free no protease
Sheep kidney cortex (powder)
Soybean trypsin inhibitor, cryst.
Steroidsulphatase
Taka diastase powder
Transacetylase (ex Clostridium kluveri)
Triphosphopyridine nucleotide
Trypsin (bovine pancreas) cryst.
Trypsinogen, 1 × cryst.
Tyrosinase (lyophilized, ex mushrooms)
Tyrosine decarboxylase
Urease
Uricase 'leo'
Xanthine oxidase

We invite your enquiries for these and other enzymes

L. LIGHT & COMPANY LIMITED

POYLE : COLNBROOK : BUCKS

DIFCO

microbiological reagents and media

for

TISSUE CULTURE

and

VIRUS PROPAGATION

These reagents are prepared and standardized to preserve unaltered the properties of the original material and including those commonly employed for the slide, roller tube and flask culture techniques for propagation and study of tissue cells and viruses *in vitro*.

REAGENTS OF ANIMAL ORIGIN—Desiccated and Liquid

Plasma, Sera and Serous Fluids
Embryos and Embryo Extracts
Ultrafiltrates

REAGENTS, CHEMICALLY DEFINED—Dilute and Concentrate

Synthetic Media—Eagle-HeLa, Eagle L, Scherer, 199, 703 and all formulas
Balanced Salt Solutions—Earle, Gey, Hanks, Osgood, Simms, Tyrode and all formulas

ENZYMES · INDICATORS · AMINO ACIDS
HYDROLYSATES · MEDIA ENRICHMENTS
BIOCHEMICALS · CARBOHYDRATES

Over 60 years' experience assures

UNIFORMITY · STABILITY · ECONOMY

Difco Laboratories

Prompt delivery from U.K stock. Write for Difco Manual and technical leaflets to the sole agents:

BAIRD & TATLOCK (LONDON) LTD., CHADWELL HEATH, ESSEX, ENGLAND

Branches in London, Manchester and Glasgow *Agents throughout U.K. and all over the world.*

BTL **complete laboratory service**

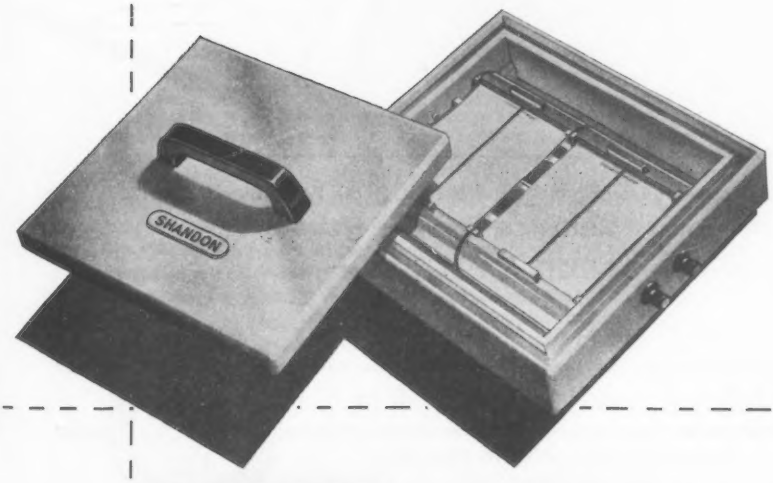
TAS/DO.2

The NEW SHANDON UNIVERSAL **ELECTROPHORESIS APPARATUS** after KOHN

Shandon's latest version of their Universal Electrophoresis tank incorporates important improvements, adding still further to the equipment's versatility, economy and convenience of operation. The bridge gap is now readily adjustable to take four strip lengths, and an improved tensioning method both simplifies the handling of thin membrane and allows a greater economy in its use.

Vapour-tight and draught-proof, the apparatus is ideal for use with cellulose acetate membrane strips. This technique, which will clearly demonstrate several fractions not seen at all on filter paper, gives sharp rapid separation—particularly of albumin. Adsorption is low and there is practically no tailing of fractions. The membrane can be made transparent to both visible and U.V. light. Only a very small sample is necessary.

Write for descriptive leaflet today.



SHANDON **SHANDON SCIENTIFIC COMPANY LTD**

6, CROMWELL PLACE, LONDON, S.W. 7, TELEPHONE KNI. 1131

Pabst RESEARCH BIOCHEMICALS

COENZYMES, COENZYME-ANALOGS AND RELATED 5′-NUCLEOTIDES

Cat. No. | Pkg. | Price

400 **COENZYME-A,** Lyophilized

CoASH (.2H_2O); $C_{21}H_{36}N_7O_{16}P_3S$ (.2H_2O)
F.W. 803

10 mg
25

SPECTRAL ANALYSIS:

		Reference Constants (8)
a_M at 260 mμ, pH 2		14.6x10³
Ratios at pH 2	250/260	0.84
	280/260	0.22

PAPER CHROMATOGRAPHY:

	R_f in Pabst Sys
R_f Observed	0.69 0.58
Approx. % by Visual U.V.	Trace 75
Indophenol Reduction (9)	+ +

ENZYME ASSAY (10):

	Theory	Observed
Lipmann Units per mg.	394	300 ± 60

300 **DIPHOSPHOPYRIDINE NUCLEOTIDE,** Coenzym

DPN (.2H_2O); $C_{21}H_{27}N_7O_{14}P_2$ (.2H_2O)
F.W. 699

100
500
1 g.
5 g.

SPECTRAL ANALYSIS:

		Reference Constants	Observed
a_M $DPN_{ox.}$ at 260 mμ, pH 7 (11)		18.0x10³	(17.7 ± 0.5)x
a_M $DPN_{red.}$ (Alc. Dehydrogenase) at 340 mμ, pH 10 (16,13)		6.2x10³	(5.9 ± 0.2)x1
Ratios $DPN_{ox.}$ at pH 7 (2)	250/260	0.83	0.83 ± 0.03
	280/260	0.22	0.22 ± 0.02

PAPER CHROMATOGRAPHY:
R_f in Pabst System No.

I		III	
Principal Spot	Trace Spots	Principal Spot	Trace Spots
0.35	0.20 (ADPR*)	0.31	None

*Adenosine Diphosphoribose

"when you can measure what you are talking about, and express it in numbers, you know something about it..."
Lord Kelvin

OUR NEW CATALOG TELLS YOU WHAT YOU WANT TO KNOW ABOUT THE PURITY OF . . .

Pabst
RESEARCH BIOCHEMICALS
IN READILY VERIFIABLE QUANTITATIVE TERMS

For similar quantitative information about all of the Pabst highly purified 5′-RIBONUCLEOTIDES, COENZYMES, COENZYME-ANALOGS, RIBONUCLEIC ACID and HYDROLYSIS PRODUCTS —

write for your copy of our 1959 catalog

PABST LABORATORIES

Division of PABST BREWING COMPANY
1037 W. McKINLEY AVE.
MILWAUKEE 5, WISCONSIN

ROCHE vitamins and related substances available for research

Synthetic Cryst. Vitamin A—Acetate, Alcohol, and Palmitate

β-Carotene cryst.

Aneurine Hydrochloride B.P. (Vitamin B_1.)

Riboflavin B.P. (Vitamin B_2.)

Nicotinic Acid B.P.

Nicotinamide B.P.

Pyridoxine Hydrochloride B.P.C. (Vitamin B_6.)

Pyridoxamine dihydrochloride

Pyridoxal-ethyl-acetal hydrochloride

Calcium-d-Pantothenate

Sodium-d-Pantothenate

d-Biotin

Cocarboxylase

Folic Acid

Inositol

Ascorbic Acid B.P. (Vitamin C.)

Sodium Ascorbate

Calciferol (Vitamin D_2.)

DL-alpha-Tocopheryl Acetate B.P.C. (pure synthetic Vitamin E.)

DL-alpha-Tocopherol (unesterified)

Disodium DL-alpha Tocopheryl phosphate

Menapthone B.P.

Pyridoxal-5'-Phosphate

ROCHE PRODUCTS LIMITED · 15 Manchester Square London W1

Telephone Welbeck 5566

Biochemistry in Relation to Medicine

Third Edition

C. W. CARTER, M.A., B.M., B.Ch.,
R. V. COXON, M.D., D.Phil.,
D. S. PARSONS, M.A., D.M.,
and R. H. S. THOMPSON, M.A., B.Sc., D.M.

In this new edition, most of the chapters have been almost entirely rewritten in order to cover some of the more important developments which have occurred in this rapidly growing subject in the last few years. The scope of the book has been extended by the inclusion of additional chapters dealing with the subjects of Digestion, Intestinal absorption and Endocrinology. In the work of revision and in the introduction of this new material the original authors have had the collaboration of Dr D. S. Parsons and Dr R. V. Coxon.

The book is designed primarily for students reading for a degree in Biochemistry, Physiology, or Medicine, but it will also be of value to postgraduate students and to workers in the field of clinical medicine.

635pp., 50s. net

LONGMANS

CHELSEA COLLEGE OF
SCIENCE AND TECHNOLOGY
MANRESA ROAD, S.W. 3

Department of Chemistry

A course of 20 postgraduate lectures on

"*BIOCHEMISTRY*"

(to be arranged in four groups) will be given by Dr M. W. Neil and Dr R. B. Rabin on Mondays 7.15 to 8.30 p.m. during the period 5 October 1959 to 21 March 1960. The course (as in previous years) is designed for students who possess a knowledge of Chemistry to degree standard. Fee: £2 for whole course or 10/- for each group. A leaflet giving details is obtainable from the Secretary.

Heterocyclic Chemistry

ADRIEN ALBERT

This comprehensive single-volume treatment of heterocyclic chemistry has largely been made possible by Professor Albert's provision of a new logical framework within which the great diversity of known facts, and later acquisitions of knowledge, can be marshalled on rational lines: the subject is divided into hetero-paraffinic, hetero-ethylenic, and hetero-aromatic chemistry, and a further sub-division of the latter on the basis of the distribution of π-electrons in the ring provides a key to the interpretation of physical and chemical properties.

Demy 8vo, 434 pages; illustrated; 45s.

THE ATHLONE PRESS
OF THE UNIVERSITY OF LONDON

THE
BIOCHEMICAL JOURNAL

Index of Authors and Subjects

1947–1957 VOLUMES 41–65

EDITED BY
F. CLARK and F. G. CROSSE

PRICE 50s.
(30s. *to members of the Biochemical Society*)

Orders to:
CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE
200 EUSTON ROAD, LONDON, N.W. 1

Carbon-14-Labelled Glucose

AT HIGH SPECIFIC ACTIVITY

Three forms of carbon-14-labelled glucose are now available at exceptionally high specific activity. They are packaged in filter-paper strips in evacuated sealed ampoules to reduce decomposition by self-irradiation, which should not exceed 2% *per annum*.

CFB.35	D-Glucose-C14 (G) (high specific activity)	40 mc/mM	(128 μc/mg)
CFA.204	D-Glucose-1-C14 (high specific activity)	15 mc/mM	(48 μc/mg)
CFA.205	D-Glucose-6-C14 (high specific activity)	15 mc/mM	(48 μc/mg)

There is no increase in price for these high specific activity sugars. The corresponding forms of labelled glucose remain available at lower specific activity (2-3 mc/mM) in conventional packages for those who prefer them.

(G)=Generally labelled

Further information will gladly be supplied by:

THE RADIOCHEMICAL CENTRE, AMERSHAM,
BUCKINGHAMSHIRE, ENGLAND.

TAS/RC39

...take the step to...

ONE COMPLETE SOURCE FOR ALL YOUR RESEARCH BIOCHEMICALS NEEDS

A COMPLETE SELECTION OF

MORE THAN 200 NUCLEOPROTEINS AND DERIVATIVES

Typical Derivatives

Adenosine Triphosphate	Uridylic Acid
Cytidine	Uridine
Cozymase	2, 6 Diamino Purine Sulfate
Coenzyme I, II, A	8 Aza Guanine
Cytosine	8 Aza Thymine
6 Mercaptopurine	Glucose-6-Phosphate

Three days after your order is received it will be delivered in most parts of Europe

NUTRITIONAL BIOCHEMICALS CORPORATION

21010 MILES AVE. • CLEVELAND 28, OHIO

Write for new Catalog, March 1959. More than 2500 items. Write Dept. 112